THE EFFECT OF PERIODIC OPERATION ON MIXED AND RECOMBINANT BACTERIAL POPULATIONS

BY

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Industry is making increased use of bacterial processes for the production of highly valued biochemicals. The transition from lab to batch reactor to industrial scale continuous operation for ordinary chemical reactions is often a difficult process, especially so for the poorly understood, barely modeled biological systems. Just as certain chemical systems benefit from continuous periodic operation so might certain classes of biological systems.

The object of this work is to determine if general mixed competitive populations of bacteria grown under continuous operation might benefit from periodic operation, for example in the event of contamination. Also as an extension of the competing populations work, the specific problem of recombinant populations and the competition that

results due to segregational instability have been examined to determine if plasmid retention can be accomplished by periodic operation.

It has been found that for systems where simple steady-state based unstructured models are valid, no periodic operation will prevent the extinction of the plasmid-bearing strain in continuous operation. However it is known that models of this type predict responses to environmental changes that are much more rapid than is seen experimentally. To take lag in response time into account, these simple models have incorporated into them a time delay which does not affect their steady-state predictions but better predicts the effects of transient operation. Using these models it was predicted that simple periodic manipulation of operating parameters such as dilution rate or inlet substrate concentration can prevent the extinction of the more slowly growing strain in either general mixed or recombinant cultures.

Experimental verification of this idea was sought using recombinant Escherichia coli containing plasmid pbr322 which confers resistance to tetracycline and ampicillin. It was demonstrated that simple square wave cycling in the dilution rate to a chemostat could indeed reverse the trend towards plasmid loss.

A method was also developed for identifying the form of delay to be incorporated into a steady-state based model using transient response information.

CHAPTER 1
INTRODUCTION

Mankind has made use of the other forms of life around him since prehistoric times. It was the mastery of agriculture that led ultimately to the development of civilization, since the assurance of a food supply gave Man the leisure time necessary to contemplate the world around him. It has been suggested by some that a chance encounter with fermented wheat was the driving force that led to the development of agriculture. In order to maintain a supply of that amazing nectar, beer, the nomadic hunter-gatherers of prehistoric earth were forced into cultivation and as a by-product civilization.

Utilization of bacterial processes has also given us bread, wine, and cheese; the basics of the world's diet. Industrial society makes increasing use of bacterial processes to produce the chemicals it needs. Most of the chemicals are not the commodity scale products often derived from the enormous petrochemical industry but smaller scale, highly valued compounds that have eluded our abilities to synthesize. The most obvious exception is the production of citric acid (1,2). Once derived almost entirely from lemons, this chemical basic to the food processing industry is now produced almost exclusively from Aspergillus niger in enormous batch fermentors.

Even some of the more common pharmaceuticals are now produced easily and on a large scale. It is engineering that has made it possible to go from test tube to 10,000 gallon fermentor production. Consider the case of penicillin, originally produced in beakers on a shake table; when it became necessary to produce it in quantity, thousands of beakers were placed on hundreds of tables, a rather cumbersome scale-up. Application of engineering to these biological processes is very important.

In applying chemical engineering principles to these complicated biological systems, it is prudent not to forget the knowledge gained from many years of scholarship in more traditional areas. It is common knowledge that chemical processes often benefit from operation other than batch or steady-state continuous in the optimization of some production objective. As biochemical systems move from test tube scale to batch and continuous fermentors, it is important to determine if they too will benefit from similar operating strategies. The fed-batch optimization in the production of penicillin took it from lab shake flasks to industrial scale batch fermentors.

Many bacterial processes are run in a batch or fed-batch manner; but as with chemical systems, there may be certain cases when a process would benefit from continuous operation. The usual benefits of capital cost savings might result as well as particular benefits that arise from growing the organisms in an environment with minimal variations in reactor environmental conditions, such as substrate concentration. If the organism is of the type that makes a growth associated product, then continuous operation could allow a productivity increase if the organism could be kept in a balanced growth mode rather than exhaust its environmental resources as would occur in batch. This would necessitate a reactor shutdown with the requisite down time while the reactor is cleaned and the inoculum culture prepared. There is also the down time that results while the culture comes up to a productive size, the lag phase it experiences before the onset of exponential growth.

No improvement in any process is without its drawbacks however. For continuous production of biological products in a CSTR, there is the ever present threat of contamination. A single contaminating organism with the appropriate growth properties has the ability not only to reduce productivity, but ultimately this contaminant can totally replace a desired organism in a reactor.

On the forefront of microbial research is the concept of genetic engineering. Application of this idea has the potential to vastly change many aspects of the production of highly desirable chemicals that have proved very difficult, if not impossible, to synthesize. Transferring the gene for the production of some desirable biochemical from a difficult-to-cultivate source to an easily grown organism promises to increase the production of these desired chemicals by many fold. These genetic transfers have proved very successful for several products such as monoclonal antibodies and human growth hormone. If these processes are carried out in batch, then it is reasonable to assume that some of them will benefit from continuous operation.

These genetically engineered strains often experience an instability problem due to the way the new genes are incorporated into the cell. Typically a new gene is given to a cell by adding the gene to a piece of extrachromosomal DNA called a plasmid. This plasmid is injected into the cell where it begins proliferating independently of the cell's own life cycle. When a cell undergoes division, these plasmids are randomly distributed among the resulting daughter cells. Occasionally a daughter cell results that does not contain the plasmid and can no longer produce the desired product. These recombinant organisms are essentially providing their own contaminant to the reactor in which they are grown and ultimately would be replaced by it.

The class of problems to be considered in this work will deal with bacterial systems where mixed populations of microorganisms compete under continuous conditions for a single nutrient source. Many types of microbial interactions exist. Among the interaction types that have been studied by others are predator-prey (3-8), commensal (9-12), symbiotic (13), and mutualistic relationships (14,15). These interactions usually exhibit steady state or cyclic behavior (autonomous oscillations) under continuous operation. The competitive interaction always results in extinction of all organisms in the culture except the most efficiently growing strain, if operated to steady state. It is desired in this work to determine if operating a chemostat in a periodic manner could prevent the extinction of a less efficiently growing organism in continuous culture, when steady-state operation is unable to do so.

Before undertaking experimental work, it is useful to determine under what conditions periodic operation might have the desired effect. The success or failure of simulations of biological system behavior would be helpful in planning experimental work, since various strategies and methods can be tested to indicate which might have the best chance of success, avoiding the tedious procedures that would be mandated by a corresponding ad hoc experimental endeavor.

To achieve this objective, it is necessary to consider the work that has been carried out in modeling of biochemical processes. The simplest models concern themselves with only the grossest features of bacterial growth, total biomass and bulk concentration of nutrient. These models are often excellent in predicting the ultimate steady-state behavior of a system, but are lacking in their ability to deal with transient situations. Since the primary goal of this work is to determine the effect of periodic operation, a higher level of model sophistication above that of the models based on simple steady-state information is essential. One way to patch this hole is to modify the latter models to account for delayed adjustment to transient environmental conditions. This is approached in this work by formulating what is known as a delay in responding to change. These delays have proved useful in predicting transient responses and in modeling bacterial interactions.

The theoretical work and the basic ideas concerning competitive mixed populations and their transient responses under periodic operation are the subject of Chapter 2. It will be shown that simple models are unable to predict the retention of the more slowly growing organism in the culture regardless of the transient strategies used. It will also be seen that the necessary model modifications (inclusion of time delay) needed to adequately predict transient responses lead to models that predict the desired behavior of preventing the extinction of the more slowly growing strain under appropriate periodic operating conditions.

Chapter 3 will contain the necessary information for the extension of the work in Chapter 2 to the more interesting and applicable case of the competitive interactions that arise when populations of plasmid containing organisms are grown in continuous culture.

The next chapter will present the results of experimental work carried out to verify the concepts discussed in Chapters 2 and 3. The system studied is a recombinant Escherichia coli containing foreign genetic material allowing it to grow in the presence of the antibiotic tetracycline. The experimental section will discuss the results of experiments carried out in batch shake flasks and batch fermentors, as well as continuous and periodic operation in a chemostat. The results of fed batch experiments will also be presented.

Finally in Chapter 5 a method is given for determining the form of the time delay in a model that best describes the behavior under experimental conditions.

CHAPTER 2
EFFECT OF PERIODIC OPERATION ON FINAL MIXED CULTURE FATE

2.1 Introduction

A substantial amount of work has been undertaken to characterize competitive mixed cultures in a chemostat (16-29). The primary result may be summarized by the competitive exclusion principle which states that competition of two microbial populations for a single limiting nutrient leads to the extinction of one population if they are grown in a spatially uniform environment subject to time invariant external forces.

If the process objective is to maintain coexistence of the mixed culture or survival of the weaker species at the expense of the stronger one, then manipulation of conventionally time-invariant process variables is one possibility that could yield the desired results. Among the process variables that could be manipulated are temperature, pH, dilution rate, and feed nutrient concentration. It has been shown experimentally that periodic variations in pH can result in a stable coexistence cycle (26). In these experiments, cycling in pH was employed to alternately benefit one species over the other.

A similar strategy could be employed using variations in feed nutrient concentration or dilution rate. The effect on mixed microbial populations of cycling in these process variables will be examined in this chapter. The theoretical development required for this study will be presented in the next section. In subsequent sections the results of numerical simulations will be presented and a system operating diagram will be constructed. A final section will discuss the results obtained as well as the areas to which these results may be applied.

2.2 Theoretical Development

Most research efforts in modeling microbial growth have resulted in models accurate in describing only steady-state operations. One set of models, termed unstructured, attempts to describe cellular growth only in terms of total cell mass or population numbers and limiting substrate concentration (30). In these unstructured models, the specific growth rate, μ, is a function of the limiting nutrient concentration, s. The usual unstructured competitive-culture model for a chemostat is expressed as follows:

$$\frac{dx_1}{dt} = \mu_1(s)\ x_1 - D\ x_1,$$

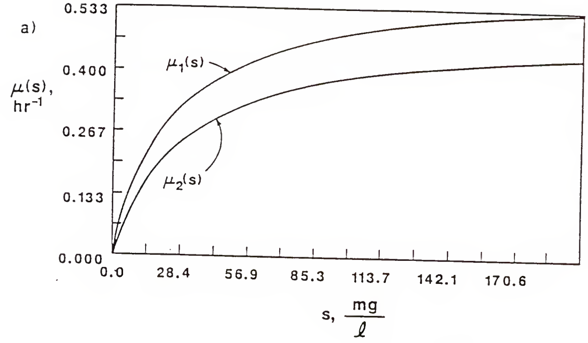
$$\frac{dx_2}{dt} = \mu_2(s)\ x_2 - D\ x_2, \qquad (2\text{-}1)$$

$$\frac{ds}{dt} = -\frac{\mu_1(s)}{Y_1}\ x_1 - \frac{\mu_2(s)}{Y_2}\ x_2 + D\ (s^0 - s),$$

where x_i is the biomass concentration of species i; D is the system dilution rate; Y_i is the yield coefficient of biomass from substrate for species i; and s^0 is the feed substrate concentration. Subscript 1 refers to the species with the higher maximum specific growth rate.

Equation system (2-1) is quite good in predicting steady-state behavior since it has inherent the assumption of "balanced growth" (30). Balanced growth implies that all cellular components are changing in the same proportion at all times and is valid only for the exponential growth phase, the phase of growth where most steady-state operations occur.

For the above model, there are two scenarios with respect to final culture fate. In the first scenario, $\mu_1(s)$ is greater than $\mu_2(s)$ for all values of s. In the second, the two growth curves intersect. A plot of specific growth rate versus substrate level illustrates the difference in Figure 2-1. In Figure 2-1a the growth rate curves do not intersect. In this case no value of either dilution rate or feed nutrient concentration will favor species 2 enough to bring about coexistence or extinction of species 1, the



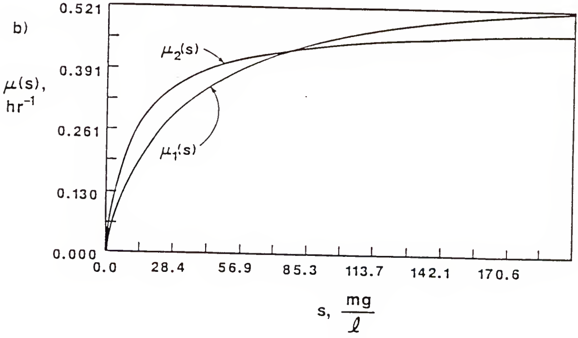


Figure 2-1. Specific growth rate for each species as a function of substrate concentration: (a) nonintersecting growth curves and (b) intersecting growth curves.

faster growing organism. In the second case (Fig. 2-1b) it is fortunate that proper selection of D and s^0 values can affect the final culture fate at will. Stephanopoulos et al. (22) examined this case and found that by periodic manipulation of dilution rate and/or substrate concentration species coexistence was possible. This chapter will concern itself with the possibility of using some transient mode of operation to modify the final culture fate in the case of Figure 2-1a.

It can be easily seen that for model (2-1) no strategy of cycling in substrate level or dilution rate will yield a washout of the faster growing species nor is coexistence possible. The proof is as follows. Consider the biomass equations in (2-1). Dividing each equation by the appropriate biomass concentration yields

$$\frac{d}{dt} \ln x_1 = \mu_1(s) - D, \qquad (2\text{-}2)$$

$$\frac{d}{dt} \ln x_2 = \mu_2(s) - D. \qquad (2\text{-}3)$$

Subtraction of eq. (2-3) from eq. (2-2), rearrangement, and integration of the result yields

$$\ln \frac{x_1}{x_2} = \int_0^\infty [\mu_1(s) - \mu_2(s)]\, dt. \qquad (2\text{-}4)$$

In order for washout of species 1 to occur, the right hand side of eq. (2-4) must approach negative infinity at large times. This is impossible for the first scenario (Fig. 2-1a), since $\mu_1(s) > \mu_2(s)$ for all s. Therefore the right hand side of eq. (2-4) is always positive. Coexistence is also excluded by similar reasoning.

Unstructured models predict a much faster response to step changes in D or s^0 than is experimentally observed (31,32). It is well known that microorganisms undergo changes in their metabolism as environmental conditions alter. They require time to adjust their enzyme levels to those needed for optimal growth under the newly imposed conditions. This time lag can be taken into account by making use of an "adaptability" parameter, α. One way to accomplish this is to let the specific growth rate be a function of z, a weighted average of previous substrate concentrations, instead of the current substrate concentration. This weighted average is defined as follows (33):

$$z = \int_{-\infty}^{t} s(\tau)\ F(t - \tau)\ d\tau \qquad (2\text{-}5)$$

where

$$F(\tau) = \alpha\ e^{-\alpha\tau} \qquad (2\text{-}6)$$

is a memory function.

This assumption leads to a model of the following form (34):

$$\frac{dx_1}{dt} = \mu_1(z_1)\, x_1 - D\, x_1,$$

$$\frac{dx_2}{dt} = \mu_2(z_2)\, x_2 - D\, x_2,$$

$$\frac{ds}{dt} = -\frac{\mu_1(s)}{Y_1}\, x_1 - \frac{\mu_2(s)}{Y_2}\, x_2 + D\,(s^0 - s), \qquad (2\text{-}7)$$

$$\frac{dz_1}{dt} = \alpha_1(s - z_1),$$

$$\frac{dz_2}{dt} = \alpha_2(s - z_2).$$

A small α value implies a lengthy response time to variations in external conditions while a large α value indicates a shorter response time. In the limit as the adaptabilities α_i go to infinity, model (2-1) is recovered. Differences in adaptability are taken into account by the relative values of α_1 and α_2. It is this difference in response times that can be exploited to modify the final culture fate. Consider the case where species 1 responds rapidly to environmental changes and species 2 responds slowly. If cycling in substrate concentration occurs rapidly enough between a large and small s^0 value, then species 2 essentially sees an average substrate concentration at all times whereas species 1 adapts itself quickly and on the average may have a lower effective growth rate than species 2. This effect is illustrated in Figure 2-2. It is this lowering of the average effective growth rate of species 1 below that of species 2 that can lead to the ultimate survival of the slower growing species at the expense of the faster growing one. Cyclic variation of dilution rate can alternately be used, since such cycling again leads to periodic variation of the substrate concentration inside the chemostat.

2.3 Simulation Results and Construction of Operating Diagram

Numerical simulations were carried out in order to construct an operating diagram which indicates the conditions under which final culture fate modification is possible. To reduce the number of parameters, the system equations were made dimensionless. By assuming a Monod expression for the specific growth rate and defining the following dimensionless quantities:

$$\begin{gathered} s' = s/s_R,\ s^{0'} = s^0/s_R,\ \beta = \alpha_1/\alpha_2,\ t' = t\,\alpha_2, \\ \mu'_{mi} = \mu_{mi}/\alpha_2,\ D' = D/\alpha_2,\ x'_i = x_i/Y_i\, s_R, \qquad (2\text{-}8) \\ z'_i = z_i/s_R,\ K'_i = K_i/s_R. \end{gathered}$$

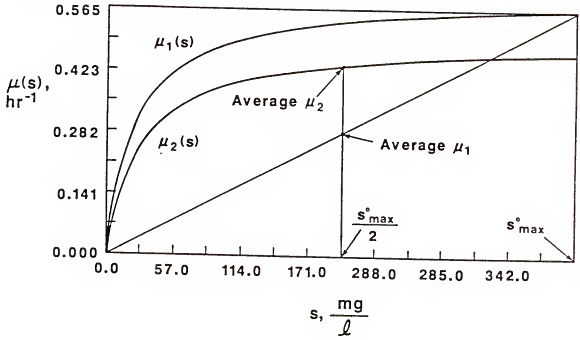


Figure 2-2. Effect of cycling in feed substrate concentration on effective growth rate. The ideal case is where species 1 reacts instantaneously and species 2 reacts very slowly. For sufficiently large s^0_{max}, effective growth rate of species 1 can become less than that of species 2.

eq. (2-7) becomes

$$\frac{dx_1'}{dt} = \frac{\mu_{m1}' z_1'}{K_1' + z_1'} x_1' - D' x_1',$$

$$\frac{dx_2'}{dt} = \frac{\mu_{m2}' z_2'}{K_2' + z_2'} x_2' - D' x_2',$$

$$\frac{ds'}{dt} = - \frac{\mu_{m1}' s'}{K_1' + s'} x_1' - \frac{\mu_{m2}' s'}{K_2' + s'} x_2' + D (s^{0'} - s'), \qquad (2\text{-}9)$$

$$\frac{dz_1'}{dt} = \beta (s' - z_1'),$$

$$\frac{dz_2'}{dt} = s' - z_2'.$$

The parameters μ_{mi} and K_i are the usual Monod constants; μ_m is the maximum specific growth rate; and K_i is the substrate saturation constant. The constant s_R is defined as the least value of feed substrate concentration at which the extinction of species 1 is possible via cycling and is derived from the inequality

$$\frac{1}{2} \frac{\mu_{m1} s_R}{K_1 + s_R} \leq \frac{\mu_{m2} s_R/2}{K_2 + s_R/2} \qquad (2\text{-}10)$$

(average growth rate of species 1 assuming infinite adaptability is equal to growth rate of species 2 at the average substrate level). Rearrangement of this inequality yields the following expression for s_R:

$$s_R = 2(\mu_{m1}K_2 - \mu_{m2}K_2)/(2\mu_{m2} - \mu_{m1}). \qquad (2\text{-}11)$$

Because this study is concerned only with case 1 (non-intersecting specific growth rates), there is an additional constraint on the Monod parameters. This constraint will prevent intersection of the growth curves and is expressed as

$$\frac{\mu_{m2}}{\mu_{m2}} > \frac{K_1}{K_2}. \qquad (2\text{-}12)$$

The feed substrate concentration was varied periodically in a square wave. During half of the period s^0 had a large value, s^0_{max}, and during the other half, it was zero.

In numerical simulations it was found that four variables are of decisive importance: the adaptability ratio, the cycling frequency, the dilution rate, and the maximum inlet substrate concentration. The ß parameter is important since the difference in adaptability is what makes survival of species 2 possible. Any ß value greater than

1.0 should bring about the extinction of species 1 provided s^0_{max} is sufficiently large, but smaller ß's require longer times for extinction to occur.

The cycling frequency is also very significant and again this is no surprise. It was found that frequencies of moderate value bring about the extinction of species 1. If cycling occurs too rapidly, both species react as if under steady-state conditions. If the cycling is too slow, the species have enough time to relax and again act as if at two steady states. No benefit occurs at either extreme.

It was also found that the value of the dilution rate is of significance. Again values of dilution rate on either extreme fail to yield survival of species 2. If the dilution rate is too small, all available substrate is consumed immediately and no cycling difference occurs upon cycling.

For a given ß it is possible to construct an operating diagram. One such operating diagram is presented in Figure 2-3. In this diagram it can be seen which combinations of dilution rate and cycling frequency lead to the extinction of species 1. As is seen in Figure 2-3, the region for extinction of species 1 is a closed region the boundary of which corresponds to coexistence of the two competing species. Figures 2-4 and 2-5 illustrate choices of dilution rate and cycling frequency from the operating diagram that lead to extinction of each species. In Figure 2-4 cycling

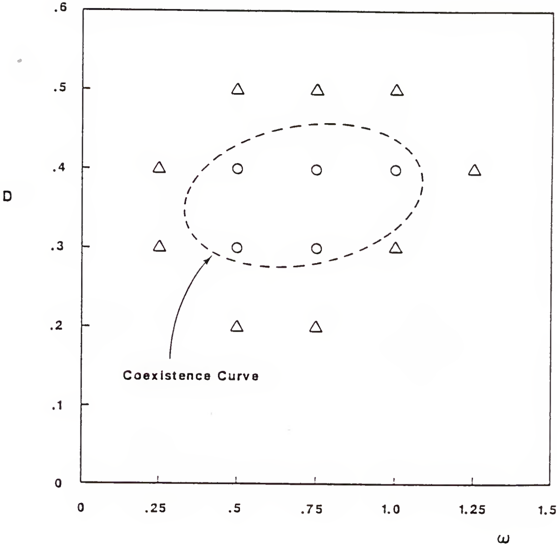


Figure 2-3. Operating diagram illustrating regions of species extinction. The symbols indicate species 2 extinction, triangles, species 1 extinction, circles, and approximate location of the coexistence curve, line.

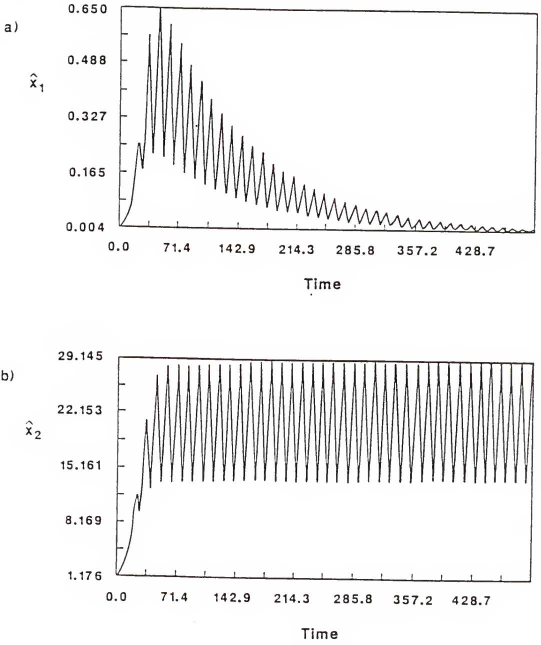


Figure 2-4. Diagrams illustrating operating parameters leading to extinction of species 1. Parameters: dilution rate = 0.4, frequency = 0.5. Shown are (a) species 1 biomass concentration versus time and (b) species 2 biomass concentration versus time.

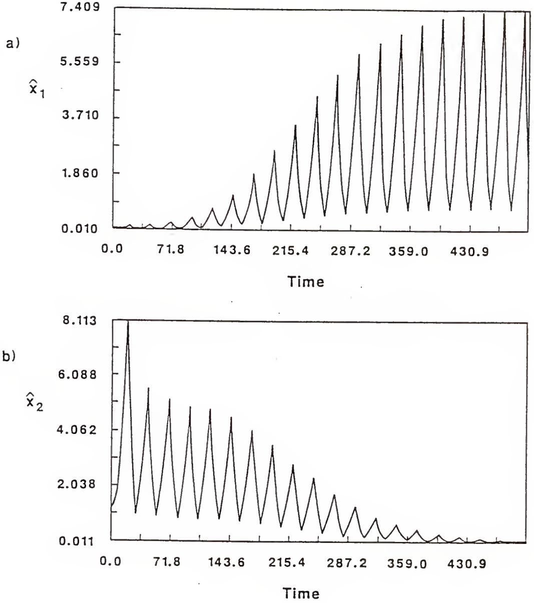


Figure 2-5. Diagrams illustrating operating parameters leading to extinction of species 2. Parameters: dilution rate = 0.4, frequency = 0.25. Shown are (a) species 1 biomass concentration versus time and (b) species 2 biomass concentration versus time.

in feed substrate concentration and proper selection of operating parameters cause the washout of species 1. In Figure 2-5 cycling again occurs, but the operating parameters selected preclude the desired extinction of species 1.

From eq. (2-11) it can be seen that s^0_{max} must be greater than s_R for extinction of species 1 to be possible. However, this is not a sufficient condition for the extinction of species 1. For large ß values, s^0_{max} can be quite close to its lower limit and still bring about the desired extinction. For smaller ß's, s^0_{max} must be significantly larger than s_R. The s_R constraint results from the ideal case where species 1 adapted instantaneously and species 2 does not adapt at all. This case corresponds to infinite ß. As ß decreases, deviation from this ideal case occurs and larger s^0_{max} values are required to produce the same behavior. The size of the region on the operating diagram where extinction of species 1 occurs increases as s^0_{max} increases.

Cycling in the system dilution rate was also examined. Square waves in dilution rate between 0.0 and D_{max} were implemented and again the adaptability difference, the cycling frequency, the maximum dilution rate, and the inlet substrate concentration were important. These variables remain significant for the same reasons as before. It is again possible to construct an operating diagram that shows where extinction of species 1 occurs. The washout of species 1 via cycling of dilution rate requires much larger s^0 values than in the case of cycling in the feed substrate concentration for the Monod model with the particular parameters chosen. In fact, the required s^0 values were so large that substrate inhibition would most likely become a significant effect. For the set of parameters employed in the simulations, the cycling in s^0 would be more economical to implement since less than half of the critical nutrient would be required as compared to cycling in D.

The delay model of eq. (2-7) is not the only way to formulate a time lag in microorganism metabolism. One alternate model, devised by Wang and Stephanopoulos (35), places the delay in the specific growth rate instead of in the substrate. This model has the form

$$
\begin{aligned}
\frac{dx_1}{dt} &= \mu_1 x_1 - D x_1, \\
\frac{dx_2}{dt} &= \mu_2 x_2 - D x_2, \\
\frac{ds}{dt} &= \frac{-\mu_1}{Y_1} x_1 - \frac{\mu_2}{Y_2} x_2 + D(s^0 - s), \qquad (2\text{-}13) \\
\frac{d\mu_1}{dt} &= \alpha_1 \left(\frac{\mu_{m1} s}{K_1 + s} - \mu_1 \right), \\
\frac{d\mu_2}{dt} &= \alpha_2 \left(\frac{\mu_{m2} s}{K_2 + s} - \mu_2 \right).
\end{aligned}
$$

While this model does predict steady-state behavior identically to model (2-7), it has a much less dramatic response to step changes in s^0. Preventing the extinction of the slower growing species was predicted with this model also.

2.4 Discussion and Conclusions

The objective of this chapter was to determine if the final fate of a competitive culture can be controlled by cycling in feed nutrient concentration or dilution rate when this is not possible for any steady-state operation. It was found that a typical unstructured non-delay model fails to predict success of this objective. However, by introducing delay it is possible to obtain differences in the species' effective growth rates that can lead to extinction of the faster growing species. Simulations indicate that for reasonable delay parameters, the final mixed culture fate can indeed be modified. Using the simulations as a basis, an operating diagram was prepared for cycling in feed substrate concentration.

This work shows relevance in several areas. First, cycling could be applied to pure continuous cultures as a method to control contamination. At first detection of an unwanted microorganism, cycling could begin between zero and some large feed substrate concentration or dilution rate.

If the most common contaminants could be identified a priori, then predetermined operating diagrams could be set up. Once the major contaminant has been identified, a standard predetermined cycling strategy could be implemented to avoid contamination.

The most important application has to do with the possibility of stabilizing the populations of genetically engineered microorganisms. These organism are generally altered by addition to the cell of a plasmid coding for the desired properties. However, these plasmids that are tailor-made to specific industrial goals often cause the organism to be put at a disadvantage to the unengineered strain. This disadvantage comes from lowering the effective growth rate since cellular resources are used for expressing the plasmid genes. This work demonstrates the possibility of maintaining an engineered population by cycling. Chapter 3 will focus particularly on the engineered organisms and their stability problem.

CHAPTER 3
EFFECT OF CYCLING ON THE STABILITY OF PLASMID-BEARING MICROORGANISMS IN CONTINUOUS CULTURE

3.1 Introduction

The advent of genetic engineering has opened a doorway to the possibility of using microorganisms to produce in quantity the exotic biochemicals that were practically unobtainable before. Presently the method of choice in creating these engineered strains is the insertion into the cell of a microorganism a small portion of extra chromosomal DNA known as a plasmid. These plasmids carry the instructions needed to provide the microorganism with some desired property. These properties include antibiotic resistance, colicin production, and increased metabolic activities (36-44).

Study of these engineered strains has uncovered a major stumbling block to large-scale industrial applications (45-51). The engineered microorganisms have a plasmid stability problem, where stability is defined as "the ability of transformed cells to maintain plasmid unchanged during their growth, manifesting their phenotypic characteristics" (36).

The tendency of the cell to lose its engineered properties is the result of nonuniform plasmid distribution during cell division. Methods to combat this problem have been suggested (38-40,42). Among these are the utilization of temperature-dependent mutants, selective environmental pressures such as antibiotics, and recombination of deficient strains.

When these strains are grown in continuous culture, a mixed population of engineered and wild type strains results. Experimental studies (36) indicate that the engineered strain is almost always slower growing than the wild type. When this fact is coupled with the competitive exclusion principle the result is the extinction of the engineered strain. One solution to this problem involves giving the plasmid an antibiotic resistance gene while growing the culture in said antibiotic. The wild types resulting from plasmid loss will be sensitive to the antibiotic and will not grow. Selective recycle (52) and feedback control (53) are among the other schemes that have been proposed as solutions.

This chapter will examine the possibility of maintaining a plasmid-bearing population in continuous culture by exploiting a difference in the adaptation times of the engineered and wild type strains to changing environmental conditions. Cycling in inlet substrate concentration will be used to illustrate the adaptation difference effect. The theoretical development required for this study will be presented in the section 3.2. In subsequent sections the results of numerical simulations will be presented and system operating diagrams will be constructed. A final section will discuss the results obtained as well as the areas to which these results may be applied.

3.2 Theoretical Development

Growing an engineered microorganism continuously leads to a mixed culture of engineered and wild strains, the wild strain resulting from reversion of the engineered strain due to loss of plasmid during cell division. The general competitive mixed-culture model for a chemostat seen in Chapter 2 is appropriate for this system once the conversion of one strain into the other is taken into account. As discussed previously, models that describe cellular growth only in terms of total cell mass or population numbers and limiting substrate concentration are generally used. These models for this application would be expressed as follows:

$$\frac{dx_1}{dt} = \mu_1(s)\ x_1 + p\ \mu_2(s)\ x_2 - D\ x_1,$$

$$\frac{dx_2}{dt} = (1 - p)\ \mu_2(s)\ x_2 - D\ x_2, \qquad (3\text{-}1)$$

$$\frac{ds}{dt} = \frac{-\mu_1(s)}{Y_1} x_1 - \frac{\mu_2(s)}{Y_2} x_2 + D (s^0 - s),$$

where x_i is the biomass concentration of species i; μ_i is the specific growth rate as a function of substrate concentration s; p is the probability per generation that a loss of the engineered plasmid will occur (54); D is the system dilution rate; Y_i is the yield coefficient of biomass from substrate; and s^0 is the feed substrate concentration. Subscript 1 refers to the reverted wild type strain, while subscript 2 refers to the engineered strain.

When equations (3-1) are solved for steady-state conditions, the following relationship is obtained:

$$x_1 = \frac{p\ \mu_2(s)}{(1 - p)\ \mu_2(s) - \mu_1(s)} x_2. \qquad (3\text{-}2)$$

This seems to indicate that coexistence might be possible if $\mu_2(s)$ is sufficiently larger than $\mu_1(s)$. However several researchers (36) have shown that $\mu_2(s)$ is almost always less than $\mu_1(s)$. This is presumably due to the plasmid-derived messenger RNA and chromosomally derived mRNA competing for the cell's limited pool of enzymes, ribosomes, energy sources, and biosynthetic precursors (41). This difference in growth rates leads to negative values of x_1 for positive

x_2 values, so that no coexistence is possible at steady state.

This competition for cellular resources most likely exerts another effect on the cells as well. They require time to adjust their enzyme levels to those needed for optimal growth under the newly imposed conditions. It seems reasonable to believe that the additional demands made on the cell's chemical machinery by the presence of the plasmid will lengthen the time required for this adjustment. The time lag in adaptability should be greater for a cell harboring the plasmid than for one without it. This difference in adaptability can be used to overcome the growth disadvantage of the plasmid-bearing strain.

The nature of the plasmid instability precludes the extinction of the wild type strain under steady-state operating conditions, but it may be possible to achieve coexistence through some transient operation by exploiting the adaptability difference. It can be easily seen for model (3-1) that no strategy of cycling in substrate concentration or dilution rate will yield a coexistence of the two strains. The proof is similar to that seen in Chapter 2 for the mixed culture case. Consider the biomass equations in (3-1). Dividing each equation by the appropriate biomass concentration gives

$$\frac{d}{dt} \ln x_1 = \mu_1(s) - D + p\,\mu_2(s)\,x_2/x_1, \qquad (3\text{-}3)$$

$$\frac{d}{dt} \ln x_2 = (1 - p)\ \mu_2(s) - D\ . \qquad (3\text{-}4)$$

Subtraction of eq. (3-4) from eq. (3-3), rearrangement, and integration of the result yields

$$\ln \frac{x_1}{x_2} = \int_0^{\infty} [\mu_1(s) - \mu_2(s)\ \{1 - p\ (1 + \frac{x_2}{x_1})\ \}\ ]\ dt. \qquad (3\text{-}5)$$

In order for coexistence to occur, after some transient time the integral in eq. (3-5) must equal zero over a cycling period. Given the constraint that $\mu_1(s) > \mu_2(s)$, then coexistence requires

$$p\ [1 + \frac{x_2}{x_1}\ ] < 0, \qquad (3\text{-}6)$$

which for positive biomass concentrations is clearly impossible.

The time lag in cellular adjustment can be taken into account by making use of an "adaptability" parameter, α. One way to accomplish this is to let specific growth rate be a function of z, a weighted average of previous substrate concentrations, instead of the current substrate concentration. This weighted average is defined as follows (33):

$$z = \int_{-\infty}^{t} s(\tau)\ F(t - \tau)\ d\tau, \qquad (3\text{-}7)$$

where

$$F(\tau) = \alpha \exp(-\alpha\ \tau) \qquad (3\text{-}8)$$

is a memory function. The time lag can also be used to lessen the reported discrepancy in the predicted response time to step changes in experimental observations (31,32).

The weighted substrate concentration assumption leads to a model of the following form (34):

$$\begin{aligned}
\frac{dx_1}{dt} &= \mu_1(z_1)\ x_1 - D\ x_1 + p\ \mu_2(z_2)\ x_2\ , \\
\frac{dx_2}{dt} &= (1 - p)\ \mu_2(z_2)\ x_2 - D\ x_2, \\
\frac{ds}{dt} &= \frac{-\mu_1(s)}{Y_1}\ x_1 - \frac{\mu_2(s)}{Y_2}\ x_2 + D\ (s^0 - s), \qquad (3\text{-}9) \\
\frac{dz_1}{dt} &= \alpha_1\ (s - z_1), \\
\frac{dz_2}{dt} &= \alpha_2\ (s - z_2).
\end{aligned}$$

As with the mixed culture case, a small value of α implies a lengthy response time to variations in external conditions while a large α value indicates a shorter response time. In the limit as the adaptabilities α_i go to infinity, model (3-1) is recovered. Differences in adaptability are taken into account by the relative values of α_1 and α_2. As mentioned above, it is this difference in response times that can be exploited to modify the final culture fate.

It was seen in Chapter 2 that for pure mixed culture competition, cycling in substrate concentration can cause the normally faster growing strain to have a lower average effective growth rate than the usually slower growing strain. It is possible that the effective growth rate difference between the engineered and wild type strains is sufficient to overcome the additional term that results from the loss of plasmid in eq. (3-5),

$$\mu_2(s)\ p\ [1 + \frac{x_2}{x_1}], \tag{3-10}$$

so that coexistence of the two strains is obtained. Cyclic variation in dilution rate can alternately be used, since such cycling again leads to periodic variation of the substrate concentration inside the chemostat.

5.3 Simulation Results and Construction of Operating Diagram

Numerical simulations were carried out in order to construct an operating diagram indicating the conditions under which coexistence of the engineered and wild type strains results. The model in eq. (3-9) was rewritten in terms of dimensionless variables to reduce the number of parameters and a Monod expression for the specific growth rate was assumed. These assumptions lead to the following model:

$$\frac{dx_1'}{dt} = \frac{\mu_{m1}' \, z_1'}{1 + z_1'} \, x_1' - D' x_1' + p \, \frac{\mu_{m2}' \, z_2'}{1 + z_2'} \, x_2' \, ,$$

$$\frac{dx_2'}{dt} = (1 - p) \, \frac{\mu_{m2}' \, z_2'}{1 + z_2'} \, x_1' - D' x_2' \, ,$$

$$\frac{ds'}{dt} = \frac{-\mu_{m1}' \, s'}{1 + s'} \, x_1' - \frac{\mu_{m2}' \, s'}{1 + s'} \, x_2' + D' (s^{0'} - s') , \qquad (3\text{-}11)$$

$$\frac{dz_1'}{dt} = \beta \, (s' - z_1') \, ,$$

$$\frac{dz_2'}{dt} = s_2' - z_2' .$$

The dimensionless parameters are defined as follows:

$$s' = s/K,\ s^{0'} = s^0/K,\ \beta = \alpha_1/\alpha_2,\ t' = t\,\alpha_1, \qquad (3\text{-}12)$$

$$\mu'_{mi} = \mu_{mi}/\alpha_2\ ,\ D' = D/\alpha_2\ ,\ x'_i = x_i/YK,\ z'_i = z_i/K.$$

The parameters μ_{mi} and K, from the Monod model, are the maximum specific growth rate and the substrate saturation constant, respectively. It is assumed that the values for parameters K and Y are the same for both strains. The only other constraint on the system is that

$$\mu_{m1} > \mu_{m2}\ . \qquad (3\text{-}13)$$

When a mixed culture is grown under constant environmental conditions, it is easy to determine exactly what the final fate is and when it occurs. When cycling in environmental conditions and looking for a coexistence of species, the final fate becomes more difficult to ascertain. In order to compare the effect of each parameter it was necessary to define a final "what" and "when" suitable to this purpose. An ultimate biomass ratio, R, was defined as

$$R = \frac{1}{T} \int_0^T \frac{x_2(t)}{x_1(t) + x_2(t)}\, dt, \qquad (3\text{-}14)$$

where T is the length of the cycling period. Because of the oscillatory nature of the biomass populations, R is meaningless unless defined at the same point in each cycle. The value of R was calculated starting at t = 0 and at all integer multiples of period thereafter. After all transient behavior has died out, R will be a constant indicating a quasi steady state has been achieved.

Square waves in the substrate feed concentration were used to produce the desired oscillations in growth rate. During half the cycle the feed substrate value was at a concentration of s^0_{max} and during the other half at zero.

All of the parameters in model (3-10) have an effect on final culture fate and keeping R as large as possible seems to be a sensible objective. To this end it was found that the relative value of the maximum growth rates was a significant limiting factor to achieving coexistence. It was seen in the literature (36) that values of μ_{2m}/μ_{1m} as low as 0.77 have resulted from the addition of plasmid to certain bacterial strains. Most of the resulting values were greater than 0.9 which is fortunate since the larger μ_{2m}/μ_{1m} values can be accommodated by the cycling of interest if certain dilution rate restrictions are taken into account. An illustration of this effect is seen in Figure 3-1. Here it is shown that μ_{2m}/μ_{1m} values as low as 0.88 can yield coexistence if the dilution rate is suitably chosen.

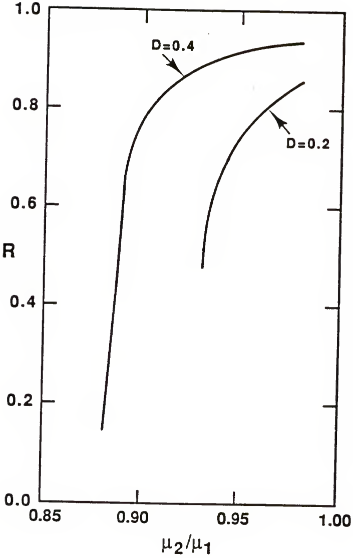


Figure 3-1. Effect of relative maximum growth rate and dilution rate on ultimate biomass ratio.

Not surprising is the fact that the largest μ_{2m}/μ_{1m} values lead to the greatest R values.

As with the pure mixed culture case of Chapter 2, it was found that values of dilution rate and period at either large or small extremes will lead to extinction of the slower growing strain. The reason the extremes do not give the desired result are the same as before. If the cycling is too rapid, both cell types react as if at steady state; if cycling is too slow, the necessary effective growth rate difference does not occur as both species have sufficient time to relax to their steady-state values. The same is true for dilution rate. However, an optimal value of both of these parameters exists with respect to R. This effect is seen in Figures 3-2 and 3-3.

The effect of s^0_{max} on R was also examined in this study. It was found that larger s^0_{max} concentrations give larger R values but there appears to be a limiting R value that is a function of the wild type reversion rate. As this point is approached large increases in s^0_{max} appear to change R very little. The existence of the optimal dilution rate becomes much more pronounced as the s^0_{max} value decreased. These effects can be seen in Figure 3-3. The increase in R and its limiting value with respect to s^0_{max} are not unexpected. If one considers the Monod growth curves, then clearly under cycling $\mu_1(s)$ will approach $\frac{1}{2}\ \mu_{m1}$ as s^0_{max} approaches large values. At the same time, effective $\mu_2(s)$

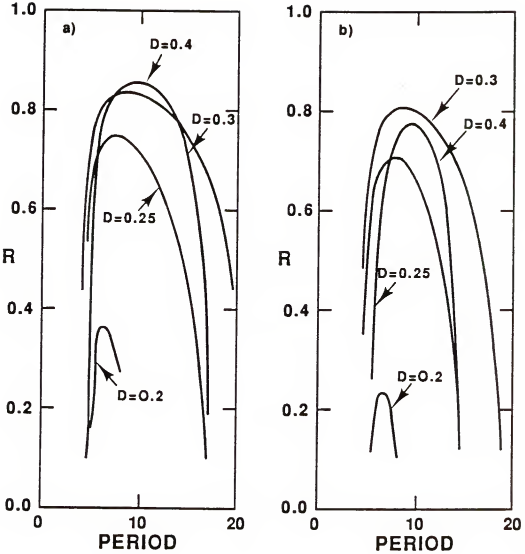


Figure 3-2. Effect of cycling period and dilution rate on ultimate biomass ratio. (a) s^0_{max} = 1000, (b) s^0_{max} = 750, (c) s^0_{max} = 500.

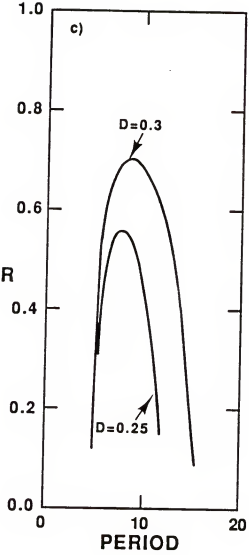


Figure 3-2--continued.

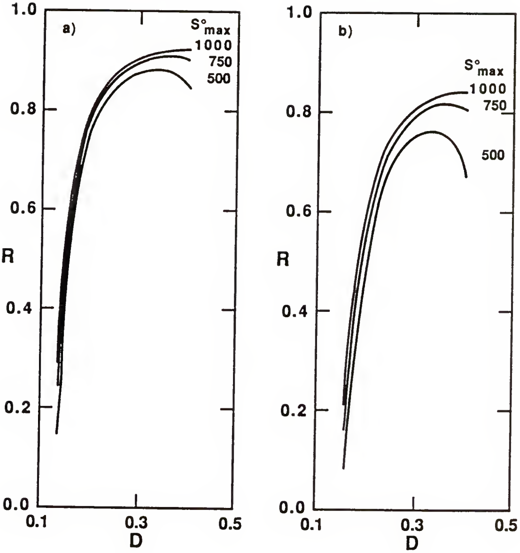


Figure 3-3. Effect of dilution rate and maximum feed substrate concentration on ultimate biomass ratio: (a) p = 0.01, (b) p = 0.02 (c) upper R and minimum s^0_{max} values being approached.

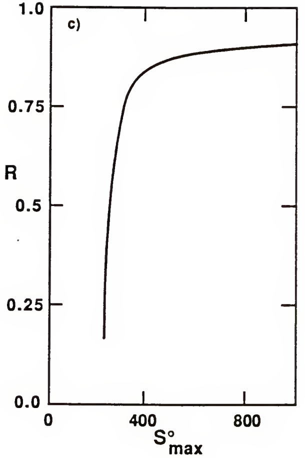


Figure 3-3--continued.

approaches $\mu_{m2}\, s^0_{max}/(2K + s^0_{max})$ and further increases in s^0_{max} make less and less difference. Together these imply that an ultimate effective growth rate difference is being approached. By the same reasoning a lower bound on s^0_{max} also exists. For the parameters used to generate Figure 3-3c a value of 225 with a corresponding R value of 0.163 is approximately the lower s^0_{max} value.

The effect of the reversion frequency, p, was also examined. As expected an increase in p causes the value of R to decrease. The optimal dilution rate becomes more obvious and the limiting R value decreases. In the literature a typical reversion frequency of 0.01 is seen (52). Obviously, if p is too large then maintenance of the engineered strain in the culture becomes impossible. The growth rate difference can no longer overcome term (3-10) from eq. (3-5).

The adaptability ratio, ß, is the most important factor in allowing for coexistence under cycling. If ß is 1.0 or less, coexistence is clearly impossible since a ß value of 1.0 has essentially the same effect as no delay at all. Any value of ß greater than 1.0 should yield coexistence when the effect of the other parameters is taken into account. It may take very large periods of time to achieve the ultimate periodic state for these small ß values, however.

For a given ß, p, and μ_{2m}/μ_{1m}, it is possible to construct operating diagrams in terms of the three operating parameters that are easily manipulated. Such diagrams are seen in Figure 3-4. These diagrams illustrate the regions in which coexistence occurs. It is not unexpected that the regions shrink as s^0_{max} is reduced. The closed curves surrounding the regions of coexistence correspond to lines of constant R, in this case R = 0 (extinction of the plasmid-bearing strain). Figure 3-5 illustrates the effect on R of operating both inside and outside the coexistence region. For optimization purposes it would be useful to further subdivide the operating region with other lines of constant R. Thus for any needed R ratio a set of operating conditions would be easily available.

3.4 Discussion and Conclusions

The objective of this chapter was to determine if an adaptability difference between the plasmid-bearing and non-plasmid-bearing strains could be exploited by transient operation to stabilize the engineered population. It was found that no cycling strategy could accomplish the desired end for organisms whose growth rates were adversely affected by the addition of plasmid and whose β value was 1.0 or less. However an adaptability ratio greater than 1.0 can provide the necessary growth advantage under certain transient conditions. The effect of various growth and operational parameters was examined and simulations were

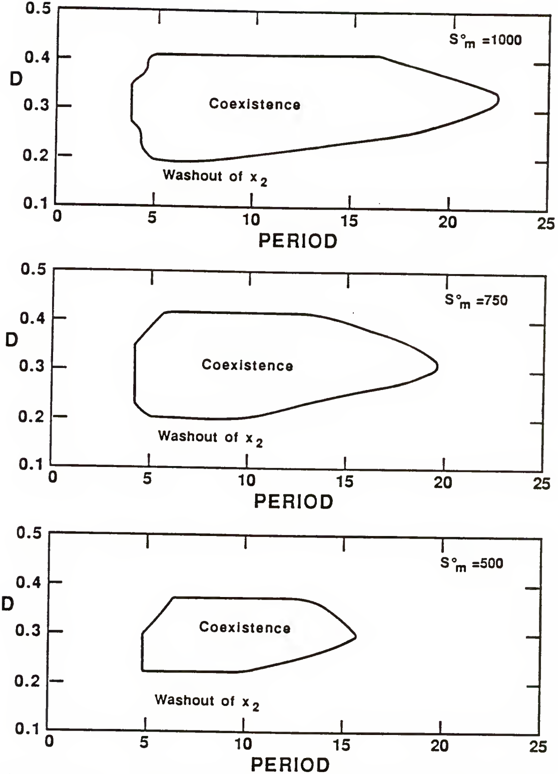


Figure 3-4. Operating diagram illustrating regions of coexistence for various s^{0}_{max} values. Line indicates approximate location of R = 0 curve.

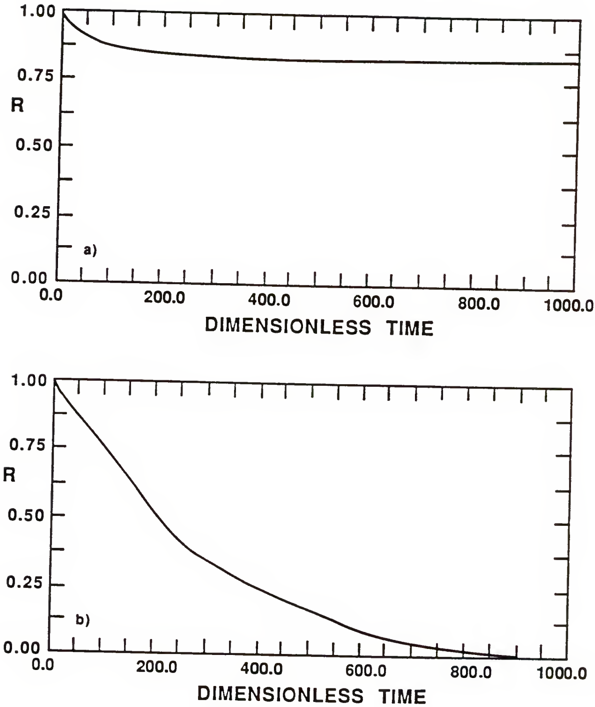


Figure 3-5. Diagram illustrating effect of operating inside and outside coexistence region on ultimate biomass ratio. (a) D = 0.3, T = 10, s^0_{max} = 1000; (b) D = 0.2, T = 15, s^0_{max} = 1000.

used to prepare operating diagrams indicating the values of dilution rate and cycling period under which the desired coexistence could occur for cycling in inlet substrate concentration.

Because of the importance that genetically engineered microorganisms will have in future industrial applications, any study dealing with improvements in their survival rate is very significant. No work has been done on measuring the relative adaptabilities of populations of engineered and wild type strains. Whenever the necessary adaptability difference does exist, a transient method of maintaining a plasmid-bearing population in continuous culture may be possible. This transient operation has the virtues of simplicity and economy of operation. Such methods might well replace the use of antibiotic selection pressures to restrict the growth of the wild strain. The use of antibiotic is quite costly on an industrial scale.

In the next chapter a recombinant Escherichia coli strain harboring a plasmid conferring antibiotic resistance to the culture will be used to experimentally verify the results of this chapter. Batch experiments will be undertaken to determine if the recombinant strain and its wild counterpart have the necessary properties to expect a reversal in the trend of plasmid loss from the culture through periodic operation. Continuous experiments will demonstrate that plasmid loss with time does indeed occur.

These results will be compared with periodic operation to verify that reversing the trend towards plasmid loss is possible.

CHAPTER 4
EXPERIMENTAL BEHAVIOR OF RECOMBINANT ESCHERICHIA COLI POPULATIONS

4.1 Introduction

In Chapter 3 it was theoretically demonstrated that the extinction of the plasmid-bearing strain from a mixed population of recombinant microorganisms can be prevented by manipulating the microbial environment. This can be accomplished in those cases where the less efficiently growing strain also reacts more slowly to changes in its environment. The purpose of this chapter is to verify various aspects of microbial growth to determine if 1) the recombinant strain and its wild strain counterpart under investigation have the necessary properties that would permit them to interact in the manner previously described and 2) periodic operation of this system could indeed lead to avoidance of the undesired extinction.

Experiments were carried out to characterize the growth of the mixed recombinant system. The experiments will be discussed in turn, but first it is necessary to consider the materials and methods used. This will be the topic discussed in the next section. Subsequent sections will discuss the results of shake-flask batch, batch fermentor, fed-batch, and constant and periodic continuous experiments.

4.2 Materials and Methods

The organisms used in this study were a strain of Escherichia coli harboring the plasmid pbr322 and the host strain of E. coli from which the engineered strain was developed. Both strains were obtained from the American Type Culture Collection (ATCC), strain numbers 31344 and 31343, respectively. The plasmid contained in this organism provides the cell with resistance to the antibiotics tetracycline and ampicillin. The resistance to tetracycline will be the marker used to track the fraction of the population that contains the plasmid.

The microorganisms were grown on a mineral salts minimal medium containing glucose as its single source of carbon and energy. The medium was supplemented with two amino acids, proline and leucine, and a vitamin, thiamine, that the strains are unable to synthesize due to a metabolic deficiency. The complete medium formulation is presented in Table 4-1. For some of the early shake flask experiments, the metabolic deficiency was relieved by the addition of small quantities of nutrient broth rather than by a specific chemical addition. The inoculum was grown in nutrient broth

Table 4-1. Glucose Limited Minimal Medium

K_2HPO_4	7.0 g/l
KH_2PO_4	3.0 g/l
$(NH_4)_2SO_4$	0.1 g/l
$FeSO_4*7H_2O$	0.5 g/l
$CaCl_2$	11.1 mg/l
$MgSO_4*7H_2O$	0.1 g/l
Proline	20.0 mg/l
Leucine	20.0 mg/l
Thiamine	2.0 mg/l
Glucose	0.2-3.0 g/l

and transferred with the seed bacteria to the minimal medium shake flask. The fraction of plasmid in the population was determined by carrying out serial dilutions of samples and growing them on nutrient agar plates, both with and without the addition of tetracycline. The tetracycline concentration on the plates was maintained at a typical 12-15 μg/ml. The plates were then incubated at 37° C and allowed to grow for 36 to 48 hours. The number of colony forming units on the plates was then counted and the concentration of each type of bacteria in the original sample calculated using the dilution factor of the serial dilution.

The concentration of biomass in a sample was determined by measuring the absorbance at 550 nm in a Milton Roy Spectronic 20D spectrophotometer. It is known that optical density is proportional to cell-mass density over the range of values that occurred in this study (30). The conversion factor from optical density to dry weight of biomass was determined by filtering samples of biomass and drying them for various absorbance values. The concentration of residual glucose in a sample was measured by an Analytic Research Model 110 Glucose Monitor. The analyzer uses an immobilized enzyme membrane mounted onto a electrochemical sensor. The enzyme, glucose oxidase, catalyzes the reaction between glucose and oxygen to produce hydrogen peroxide.

The sensor detects the hydrogen peroxide and produces a signal proportional to glucose concentration.

The inoculum cultures for all experiments were prepared by culturing from nutrient agar slants the appropriate strain of bacteria. The inocula were then transferred to 250 ml seed flasks containing minimal medium and glucose at a concentration of 3.0 g/l. These flasks were then placed in a Blue M Magniwhirl constant temperature bath at 37° C. When the seed flasks become visibly turbid (usually after one day), a sample of the seed flask was used to inoculate the experimental shake flask or fermentor. The size of the inoculum varied depending on the specific experimental objective.

The shake-flask batch experiments were carried out in 500 ml beakers with a working volume of 250 ml. Depending on the duration of the experiment to be run, an inoculum between 1 and 10 ml would be added to the flask. At intervals thereafter 10 ml samples of system fluid would be taken for analysis. Experiments were usually terminated at the onset of what appeared to be stationary phase. The early shake-flask experiments were carried out prior to the acquisition of the spectrophotometer, so no biomass concentration measurements were made.

Batch experiments were most often carried out in one of two batch fermentors. These 1 and 2 l New Brunswick Multigen bench-top fermentors were temperature controlled and magnetically agitated. Baffles in the fermentor helped to ensure good mixing. The working volumes of these reactors were 0.75 and 1.5 l, respectively. After a fermentor was autoclaved and cooled, sterile minimal medium without glucose was pumped into the reactor using an HBI Multistatic pump. A sterile glucose solution was then added to the reactor. The minimal medium was sterilized separately from the glucose solution, since glucose heated in the presence of phosphate produces a toxic by-product.

The fed-batch experiments were most commonly run in parallel with a batch experiment using the same inoculum for comparison purposes. This procedure helps eliminate some of the variability that occurs due to the difficulty of having inoculum in the identical place in its growth cycle for different runs. When both fermentors were in use for the parallel experiments, the working volumes for both were 750 ml. Obviously the initial volumes of the two reactors were different, but they both received the same total quantity of inoculum.

All of the continuous experiments, those with either constant or periodic input, and some batch experiments were carried out in a 3.7 l Bioengineering KLF 2000 fermentor. The working volume of all experiments was 2 l. Good mixing was accomplished by the rotation of two flat blade agitators at 500 rpm. A baffle cage ensured even better mixing. Constant temperature of 37° C was maintained in the reactor by a PT 100 temperature sensor, a Bioengineering K54450 controller, and an 800 watt heater. An Ingold Ag/AgCl pH electrode and a Bioengineering M7832N pH controller were used to maintain a constant pH 7.0 environment by the addition of a NaOH solution.

The dilution rate of the system was maintained by a load cell that kept the volume of the reactor constant by running the harvest pump at a slightly faster rate than the feed pump. The load cell, by maintaining a constant reactor weight, adjusted the flow rate of the harvest pump by operating an on-off control strategy on it implemented by an independent microprocessor unit. During periodic operation, the feed pump was controlled by a PDP 11/23 computer which had the task of switching between the two levels of dilution rate at predetermined times.

For batch experiments carried out in the chemostat, a 200 ml inoculum would be sampled for analysis and the remainder would be pumped into the reactor peristaltically. This would be considered time zero for the experimental run. At intervals throughout the experiment, samples of reactor fluid would be withdrawn for analysis. For nonperiodic continuous experiments, a 200 ml inoculum would be sampled and pumped into the reactor and continuous operation would commence. For periodic operation, the same procedure would be followed as with nonperiodic operation, except that the continuous operational phase was run for 10 to 12 hours before periodic operation was started. This allowed the organisms time to adjust themselves to continuous operation and would make comparisons of continuous and periodic operation easier.

To get an indication of reactor behavior it was necessary to sample the experimental runs. The sample size for all procedures other than the parallel batch and fed-batch runs was 10 ml. This amount was the minimum needed to allow both serial dilutions and glucose analysis to be carried out. The serial dilutions never required more than 1 ml and the substrate analysis required 1.5 ml for each measurement. The glucose analysis was carried out three to five times for each sample and the results were averaged. Removing a sample of 10 ml from the batch runs had no effect on the system behavior since microbial growth is in no way affected by the total volume of the system. The dilution rate of the continuous runs could be affected by removing volume from the chemostat during operation, but the sample size was only 0.5% of the total volume and was considered insignificant. The only experiments where the sample size had to be closely monitored were the parallel batch/fed-batch experiments. The volume of the reactor in the fed-batch experiments was continually increasing due to the flow of medium into it. The removal of sample also affected the volume. The constantly changing volume added a dilution effect to the biomass concentration that did not have to be considered in the batch or continuous cases.

4.3 Results of Batch Experiments

The purpose of the batch runs was to determine the best conditions under which to operate the continuous and periodic experiments. This included characterizing the conditions under which various growth limitations occurred. The experiments also determined whether the recombinant E. coli system chosen would demonstrate the appropriate growth characteristics to allow the possibility of maintaining the plasmid-bearing strain in the culture.

The first series of batch experiments that were performed were carried out in a shaker bath at 37° C in 500 ml flasks. The first several batches to be considered do not contain specific amino acid and vitamin additions. The metabolic deficiency is corrected by the presence of residual nutrient broth added to the batch during inoculation of the culture.

In batch 1, illustrated in Figures 4-1 and 4-2, it can be seen that the recombinant fraction of this population had a starting value of essentially 1.0 and remained constant throughout the course of the experiment. No significant loss of plasmid occurs over the time frame in which observations were made. Therefore it is impossible to

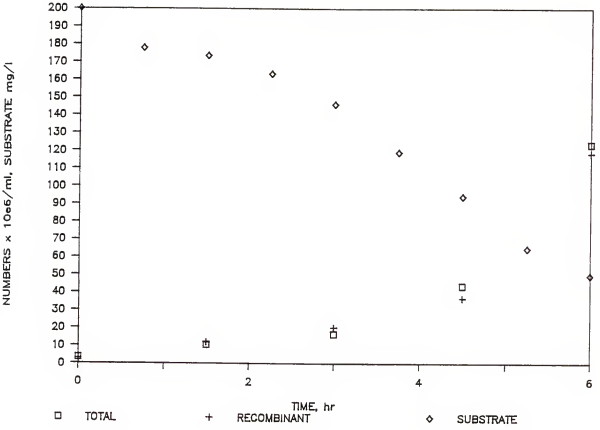


Figure 4-1. Population growth and substrate concentration for batch 1. Initial substrate concentration 200 mg/l.

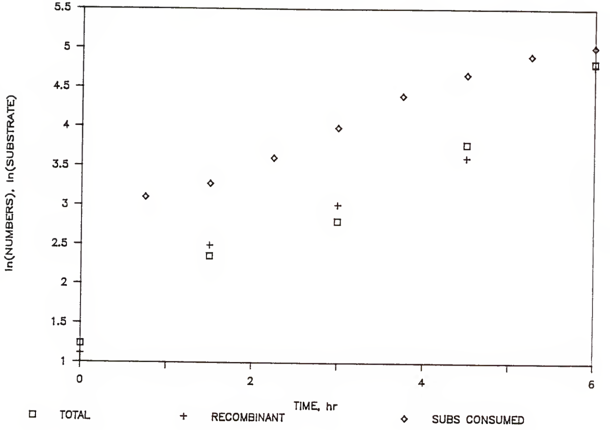


Figure 4-2. Semi-logarithmic plot of population growth and substrate concentration for batch 1.

determine if the growth rate of the recombinant is less than that of the wild type. Figure 4-2, a semi-logarithmic plot, shows that the bacteria were in their exponential growth phase over the time observed. The specific growth rate of this culture was 0.57 hr^{-1}. The specific substrate consumption rate, the rate at which substrate is removed from the medium, was 0.40 hr^{-1}. It is calculated as slope of the glucose consumption curve when plotted semi-logarithmically. These two values are not the same as is predicted for a typical Monod type batch. In the Monod case balanced growth is assumed and proportionality holds between substrate consumed and biomass produced, but this is not to be expected when growth is not balanced. This run does not show the lag phase often observed in batch growth. The starting culture must have been in a dynamic state when it was transferred from the seed flask. So balanced growth did not occur. The values of these consumption and production rates should be in agreement for a system where balanced growth does occurs. The proportionality between substrate consumed and cell number increase is very much dependent on the starting condition of the inoculum.

It should also be noted in Figure 4-1 that the culture consumed essentially all of the glucose that was available to it. The growth was limited solely by the amount of glucose initially present in the medium, 200 mg/l.

In Batch 2 (Fig. 4-3, 4-4), the rectangular plot resembles the previous one. The semi-log plot also seems familiar. Closer inspection reveals that the earliest portion of the growth curve is going through lag phase. In batch 1 the bacteria appear to adjust immediately to their new environment. The exponential growth rate for batch 2 is 1.29 hr^{-1} and the substrate consumption rate is 0.98 hr^{-1}. These values are in closer agreement than was seen in batch 1. This demonstrates the balanced growth that was expected. It also shows how important the initial state of the inoculum is. The lag phase precedes a balanced growth phase.

This run also shows another feature not seen in batch 1. The culture appears to enter stationary phase. This is expected behavior since the glucose in the medium has run out. No reversion of recombinant to wild type is detected in this batch and again no conclusions can be drawn about relative growth rates. The maximum growth rates in batch 1 and 2 were different although they were run under identical conditions. Batch 2 went through a lag phase and then into exponential growth phase. Batch 1 did not go through a lag phase and its growth rate is consequently about half the rate seen in batch 2. The culture in batch 1 did not get to balanced growth phase and does not experience as rapid a growth rate. The initial state of the inoculum is most likely the cause.

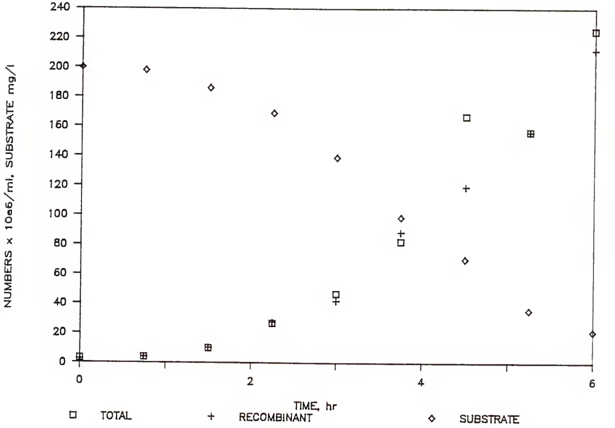


Figure 4-3. Population growth and substrate concentration for batch 2. Initial substrate concentration 200 mg/l.

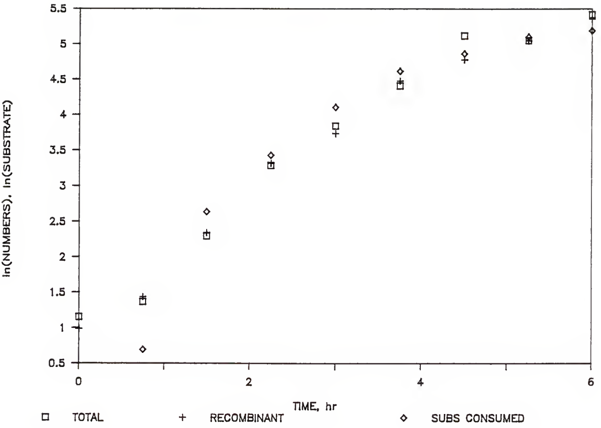


Figure 4-4. Semi-logarithmic plot of population growth and substrate concentration for batch 2.

In Figure 4-5, batch 3, the growth curves appear very similar to previous runs, but the glucose curve decreases linearly and the cultures enter stationary phase. It is important to note that in this case the initial glucose concentration is 400 mg/l, twice as large as for the two previous batches. Growth of the bacteria is arrested before the substrate is exhausted. There are two possible causes of this limitation of growth. One possibility is that for this level of initial glucose, there is another limiting substrate. Since the specific amino acid and vitamin additions were not made, these vital compounds may be limiting growth. Another cause may be the build up of some inhibitory waste product. The results of a later run appear to support the view that a nutrient other than glucose is the cause of the growth limitation.

The above three runs showed several interesting features about the growth of this culture. First, balanced growth is very dependent on the initial state of the culture. If the inoculum is in a dynamic growth state when it is transferred, then balanced growth is not observed. If a lag phase does occur upon transfer of the culture, then the specific rates of glucose consumption and population increase are comparable, as is expected in balanced growth. Also observed is the growth limitation that results when the initial glucose concentration is greater than 200 mg/l. The cause of the limitation is most likely a metabolic

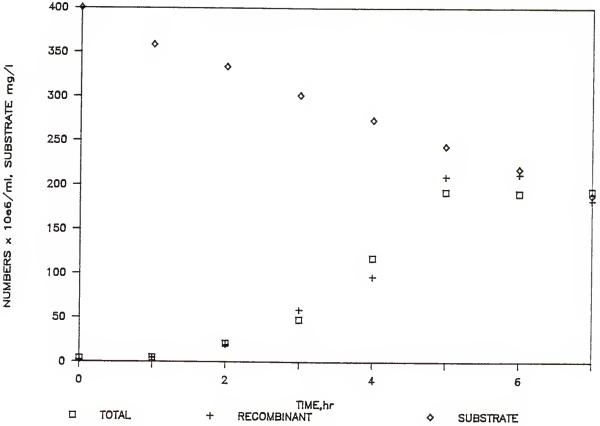


Figure 4-5. Population growth and substrate concentration for batch 3. Initial substrate concentration 400 mg/l.

deficiency. Finally, it is noted that the time frame necessary to observe the reversion of the engineered strain to wild type must be longer than the length of the batch experiments that were carried out.

The first three batches were all run at a constant temperature of 37° C, but additional batches were carried out at 42° C. This was done to determine if temperature differences might enhance the plasmid instability and make it detectable over the observed time interval. In batch 4 the first changes in recombinant fraction over the course of a run are observed. In Figures 4-6 and 4-7 this trend is readily apparent. There are two possible causes for the fractional change. The most obvious cause is reversion of the engineered strain into wild type but the growth rate difference between the two strains is the dominant effect in the evolution of the population from one type to the other. The growth rate difference is the most important effect because the original fraction of recombinants in the population is only one-third of the total. Previous batches indicate that over the observed time span, significant fractional changes are not occurring. Only when the fraction of recombinants is close to 1.0 is the reversion phenomenon important. Under these conditions reversion is the only way the wild strain is produced. After the fraction of wild types increases, then it is the growth rate difference that is responsible for further changing the

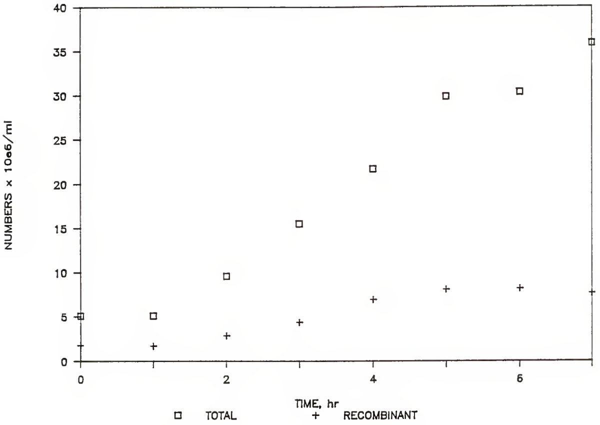


Figure 4-6. Population growth for batch 4. Temperature 42° C. Growth difference between wild and recombinant strain demonstrated.

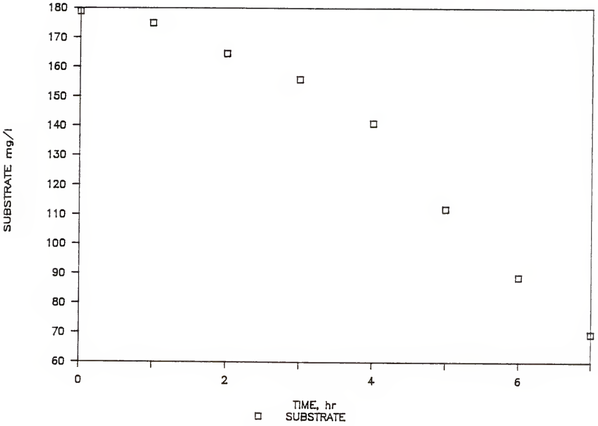


Figure 4-7. Substrate utilization for batch 4. Initial substrate concentration 180 mg/l. Temperature 42° C.

population. This run also demonstrates that the growth rates between the two strains are different. The maximum specific growth rates of the two populations are 0.63 hr^{-1} and 0.56 hr^{-1}. The substrate consumption curve yields a maximum rate of 0.51 hr^{-1}. The population increases at a much slower rate than is seen in the previous batches. This is to be expected since operation of the system at other than the optimal temperature should slow the enzyme kinetics. The glucose consumption rate is also larger for this case. It may be that the suboptimal temperature adversely affects energy conversion and hence yield, requiring more glucose consumption to produce the same result.

The rest of the shake-flask experiments included the amino acid and vitamin addition to the minimal medium rather than nutrient broth. All subsequent runs were carried out at 37° C.

In batch 5, see Figures 4-8 and 4-9, a run was carried out that clearly illustrates the existence of delay upon a change in the substrate concentration. This plot shows a batch run where starvation conditions were initially in effect. This explains the drop in population in the early portion of the curve. The only glucose in the medium was what was present due to the inoculation from the seed flask. At 2.5 hours a glucose addition was made raising the flask concentration to 1000 mg/l. The total population values

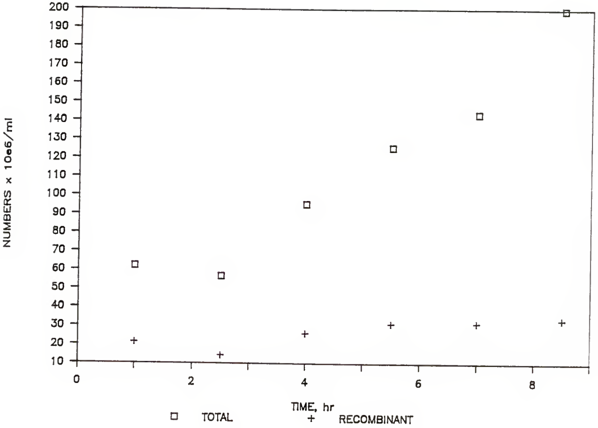


Figure 4-8. Population growth for batch 5. Total population reacts to change in substrate concentration.

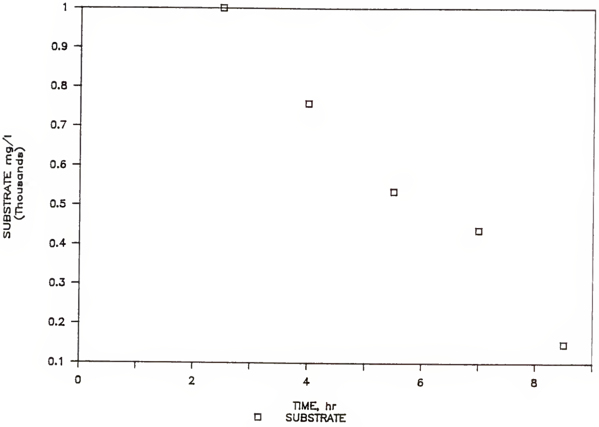


Figure 4-9. Substrate utilization for batch 5. Starvation conditions until glucose addition made at 2.5 hours.

quickly entered a rapid growth phase. The recombinant population showed almost no effect whatsoever.

This run also sheds some light on the growth limitation seen in batch 3, Figure 4-5. In that run, an initial glucose concentration of 400 mg/l resulted in growth limitation without substrate exhaustion. Two possible explanations were considered, lack of metabolites or product inhibition. The current batch had the necessary amino acid and vitamin additions and utilized all of the 1000 mg/l initial glucose concentration that it contained. This seems to indicate that the growth limitation in the previous batch was caused by metabolite deficiency.

All results from this point on will include a measure of the total quantity of biomass present, optical density. The absorbance measured at 550 nm is proportional to the amount of biomass present.

The next run, batch 6, shows that under conditions of balanced growth, the biomass concentration and population numbers increase at the same rate. Figure 4-10 shows that the exponential rates of increase for biomass and numbers were 0.72 hr^{-1} and 0.78 hr^{-1}, respectively. Also seen in this run is that growth inhibition can occur at large glucose concentrations even with the metabolite addition. The initial glucose concentration for this run was 3000 mg/l. The inhibition could be caused by one of three things: metabolite deficiency, substrate inhibition, or

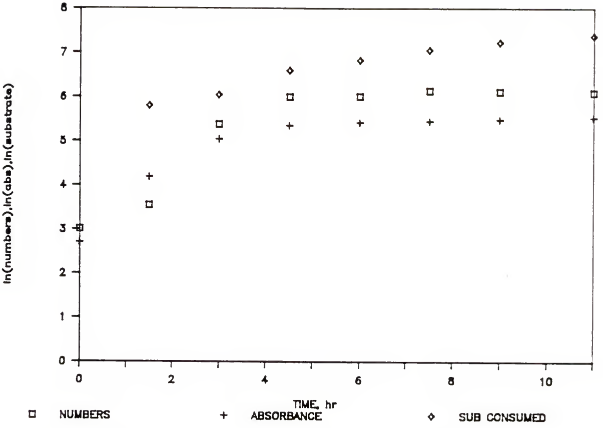


Figure 4-10. Semi-logarithmic plot of population growth, absorbance, and substrate consumed for batch 6. Balanced growth illustrated. Numbers x 10E-6, Absorbance x 1000, substrate mg/l.

product inhibition. While the previous batch indicates that metabolite deficiency is not a problem for 1000 mg/l initial glucose concentration, this result cannot be extrapolated to the larger glucose concentrations seen in the current batch. The idea of substrate inhibition has not been considered before this run since the glucose concentrations involved were relatively low. Product inhibition has been considered as a contributing factor before. There is no way to differentiate between the various growth limitation causes using the data of this run.

Another run was carried out to test the possibility of metabolite deficiency. To a run with an initial glucose concentration of 3000 mg/l was added ten times the normal amino acid and vitamin additions. This run had a slight increase in the total amount of substrate consumed, but nowhere near the levels necessary to achieve total utilization. This seems to eliminate the possibility of metabolite deficiency as the cause of the growth limitation in batch 6.

A very interesting effect is seen in Figure 4-11. In this experiment, a series of serial transfers were carried out. A 1 l batch fermentor was inoculated with a small amount of medium from a seed flask. At 24 hours, a sample from this reactor was transferred to a second reactor and allowed to grow for another 24 hours. The procedure was again carried out a third time. Samples were taken and

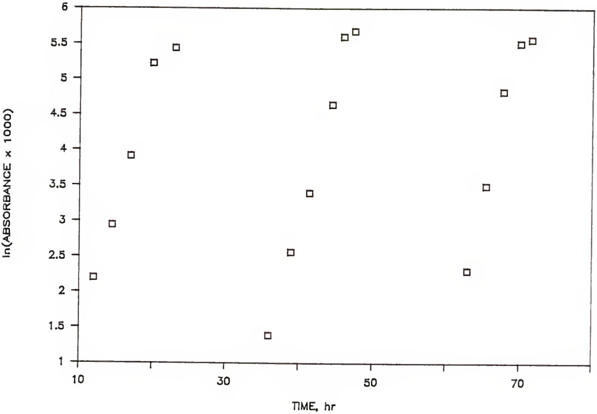


Figure 4-11. Semi-logarithmic plot of Absorbance for three runs in series. Rate of Absorbance increase changes from 0.41 to 0.59 hr^{-1} as wild strain becomes more numerous.

absorbance measured throughout the three procedures. The semi-log plot of this data shows that the effective specific growth rate is increasing over time. The value over the three runs increases from 0.41 to 0.59 hr^{-1}. This effect could possibly be a result of the reversion of recombinant into wild type, but the dominant effect is probably again the growth rate difference between the two strains.

The result of batch experiments can be summarized as follows. The amino acid and vitamin additions are necessary to prevent growth limitation if operating at an initial glucose concentration in excess of 200 mg/l. Batches with the metabolite addition and an initial glucose concentration of 1000 mg/l will grow to complete glucose utilization. Runs with an initial concentration of 3000 mg/l show a growth limitation caused either by product or substrate inhibition. A batch that starts with a lag phase experiences balanced growth. The wild strain is more responsive to environmental changes than the recombinant strain and grows at a faster rate. It appears that the chosen system has the desired properties explained in Chapter 3 that allow an attempt at improvement in recombinant retention by periodic operation.

4.4 Fed-batch Experiments

A series of parallel batch and fed-batch experiments were carried out to determine if differences in growth rate could be exploited to slow down the reversion of engineered strain into wild strain.

These experiments were carried out as follows. The fed-batch reactor was prepared with an initial amount of medium that depended on the flow rate to the reactor and the length of time the run was to be carried out. The batch reactor was filled with 1 l of medium. Then both reactors were inoculated with the same size of inoculum from a seed flask. Flow of medium into the fed-batch reactor was then started. Depending on the size of the inoculum, both reactors were sampled at regular intervals once the reactor contents were visibly turbid. The reactors were periodically sampled during the entire length of time that the fed-batch reactor was having medium added to it. This interval was dependent of the initial inoculum size. Both reactors were ultimately fed with the same total amount of medium. Both reactors continued to be sampled after the reactor feed was stopped.

The basic conclusion of this set of experiments is that there is no difference in growth rates or substrate utilization or recombinant conversion between batch and fed-batch operation for the types of fed-batch operation that were chosen in this study. A typical run is shown in Figures 4-12 and 4-13. These semi-log plots show the change in absorbance and glucose consumption that occurs for two batches, one of which was run fed-batch. The fed-batch reactor had an initial volume of 100 ml. The remaining volume was pumped in to the reactor over the next 20 hours. Additional samples of reactor fluid were taken after the flow of feed had stopped. Figure 4-13 illustrates that the glucose consumption shows the same behavior whether one is operating in a batch or fed-batch manner.

4.5 Continuous Operation

The experiments to be described in this section all took place in a chemostat with a 2 l working volume. The runs were carried out at constant temperature, 37° C, and pH, 7.0. The two control variables that were changed between runs were dilution rate and inlet substrate concentration. During a run all inputs to the system were kept constant. As discussed in Chapter 3, a system of this type at steady state would eventually exhibit extinction of the plasmid-bearing strain.

The purpose of these runs is to show that the loss of plasmid does indeed occur and the fraction of recombinants in the population decreases with time. It is necessary to

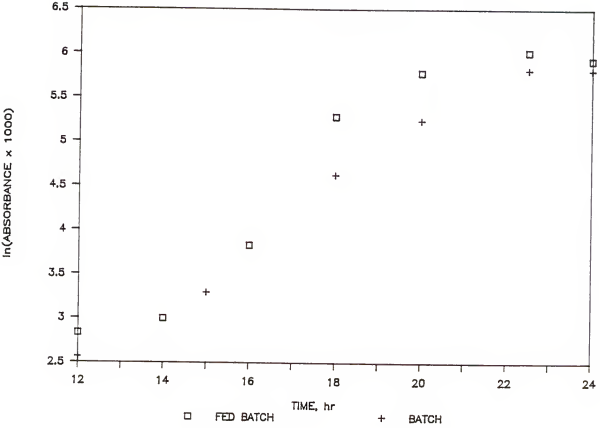


Figure 4-12. Semi-logarithmic plot of Absorbance data for a typical batch, fed-batch parallel run.

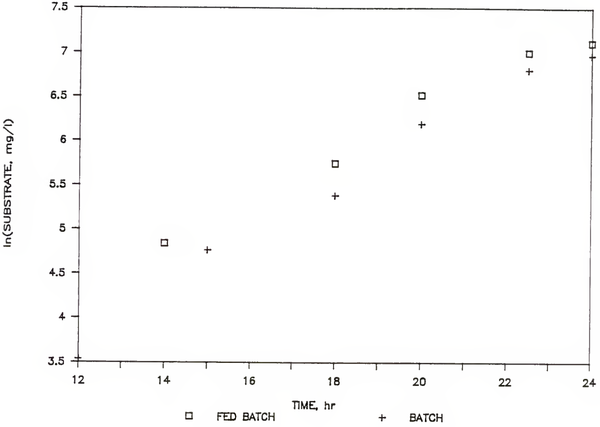


Figure 4-13. Semi-logarithmic plot of substrate consumption data for typical batch, fed-batch parallel run.

demonstrate that this problem does indeed exist for the system of interest before a remedy can be sought.

Several continuous runs were carried out to demonstrate that the mixed competitive operation does indeed result in decrease of the fraction of recombinant organisms in the culture. No true steady state was attained since a mixed population was always present in the reactor, but the values of absorbance and residual substrate concentration do seem to approach what could be considered constant values. This occurs since the two strains are identical except for the addition of the recombinant plasmid to the engineered strain. It is expected that the yield factors and other growth parameters of the two strains are sufficiently similar that only significant changes in the total fraction of the population result in actual differences in the two gross features (absorbance and residual glucose) of system behavior that are considered here.

A listing of the so called "steady" values is given in Table 4-2. The data are interesting in several regards. The five sets of data where both absorbance and residual substrate appear to be constant involve four values of the dilution rate and two values of the inlet substrate concentration. Operation at an inlet glucose concentration of 1000 mg/l results in a very little residual glucose, regardless of the system dilution rate. The two runs carried out at 2000 mg/l have residual glucose values that

Table 4-2. Apparent Steady State Values

D hr^{-1}	Ave Absorbance	Ave Substrate mg/l	Inlet Substrate mg/l
.11	.139	20.2	1000
.2	.205	18.5	1000
.25	.211	32.0	1000
.2	.242	419.0	2000
.28	.260	680.0	2000

indicate that significant substrate utilization is occurring. It is hard to draw conclusions based on the residual substrate values for the runs carried out at 1000 mg/l since they are all very close to the same value. The absorbance values are much more conclusive. A typical Monod-type model predicts that the biomass concentration decreases with increasing dilution rate. This relationship does not hold for this system. Also in typical Monod behavior, the residual substrate concentration is not affected by the inlet substrate concentration. The runs carried out at D = 0.2 hr^{-1} and different inlet substrate values show that this too is violated for this system. Obviously Monod type behavior is not in operation here.

Batch 10, see Figures 4-14 and 4-15, shows the effect on absorbance and substrate concentration of switching from batch operation to continuous with a dilution rate of 0.2 hr^{-1} and an inlet substrate concentration of 1000 mg/l. The biomass was diluted by the flow of medium through the chemostat. The reactor had a very small residual glucose concentration at the beginning of continuous operation and this value changed very little over 60 hours. At 70 hours a step change in dilution rate was made to 0.25 hr^{-1}. After an initial transient interval, the absorbance and substrate again stabilize to "steady" values. The recombinant fraction had not dropped to zero for either of these dilution rates, so the values cannot truly be called steady.

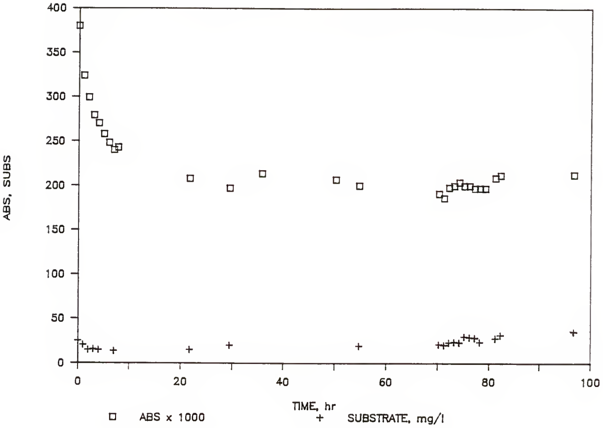


Figure 4-14. Absorbance and substrate concentrations for batch 10, continuous operation. Switched from batch operation at time zero to dilution rate 0.2 hr^{-1}. At 70 hours, dilution rate increased to 0.25 hr^{-1}. Inlet substrate concentration 1000 mg/l.

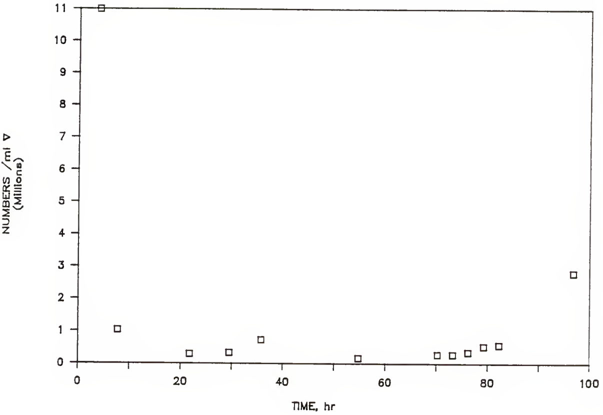


Figure 4-15. Effect of continuous operation on population, batch 10. Dilution rate 0.2 hr^{-1} until 70 hours when switched to 0.25 hr^{-1}.

Operating the system at such a low substrate concentration raises the possibility that the cell maintenance is a dominant factor.

Figure 4-16, batch 11, shows the transient response from batch operation to a dilution rate of 0.109 hr^{-1}, again at an inlet substrate concentration of 1000 mg/l. After 80 hours, the absorbance reaches another "steady" value. Again the recombinant fraction is nonzero. The low residual glucose concentrations suggest that a larger inlet concentration will be required for periodic operation to succeed. If dilution rate cycling were carried out for an inlet glucose concentration of 1000 mg/l, no significant differences in the substrate concentration in the reactor will be seen when switching between batch and continuous operation. This seems to indicate that operation at a larger inlet substrate concentration is in order.

Batch 12, see Figures 4-17 and 4-18, shows the transient response from batch to a dilution rate of 0.2 hr^{-1} and an inlet substrate concentration of 2000 mg/l. The residual glucose level for this case is much larger than in the previous batches. In this case the cell's maintenance requirements are an insignificant factor in cell growth. Again the residual substrate and absorbance values reach a "steady" value. The recombinant fraction for this case shows a definite drop over time from about 80 hours.

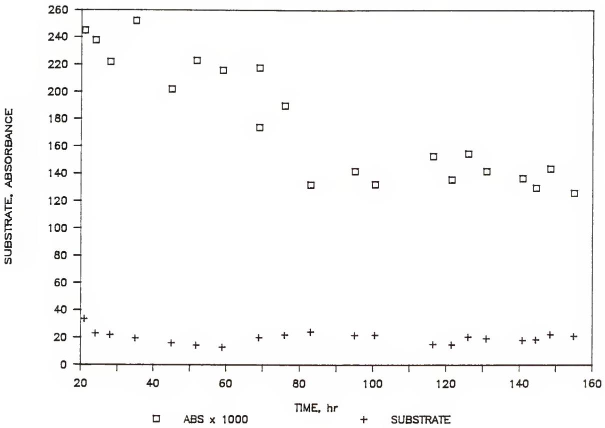


Figure 4-16. Absorbance and substrate concentrations for batch 11, continuous operation. Dilution rate 0.109 hr^{-1}. Inlet substrate 1000 mg/l.

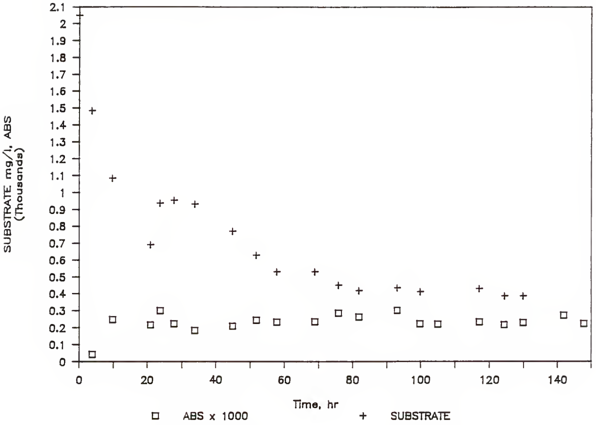


Figure 4-17. Absorbance and substrate concentrations for batch 12, continuous operation. Dilution rate 0.2 hr^{-1}. Inlet substrate 2000 mg/l.

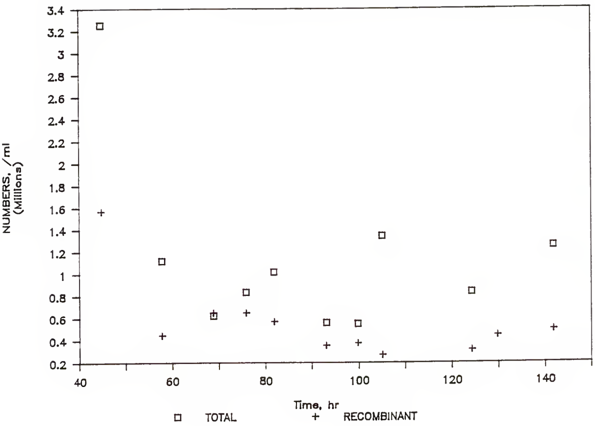


Figure 4-18. Effect of continuous operation on population, batch 12. Dilution rate 0.2 hr^{-1}.

Batch 13, see Figures 4-19 and 4-20, shows some interesting behavior. The run begins under batch conditions and then continuous operation at a flowrate of 0.28 hr^{-1} and an inlet substrate of 2000 mg/l starts. This batch shows an overshoot to the change in operating conditions. The absorbance first increases and then drops while the substrate increases to a large value and then decreases. The relatively large dilution rate increases the flow of medium through the reactor and causes the residual glucose to increase and then drop as the number of cells increases to take advantage of the extra glucose that was available. This in turn causes the substrate to drop. Again the loss of plasmid in the population with time is very clearly demonstrated.

Batch 14, Figures 4-21 and 4-22, also was run with a glucose concentration of 2000 mg/l and a dilution rate of 0.2 hr^{-1}. Both the absorbance and the glucose concentration show dynamic behavior over the interval observed. The recombinant fraction shows a definite drop. The run was stopped after 80 hours due to contamination.

Batch 15, Figure 4-23 and 4-24, was carried out at a dilution rate of 0.2 hr^{-1} and substrate concentration of 2000 mg/l. The absorbance values show the familiar "steady" behavior, but substrate increases and then begins a trend downward that continues over the entire period of

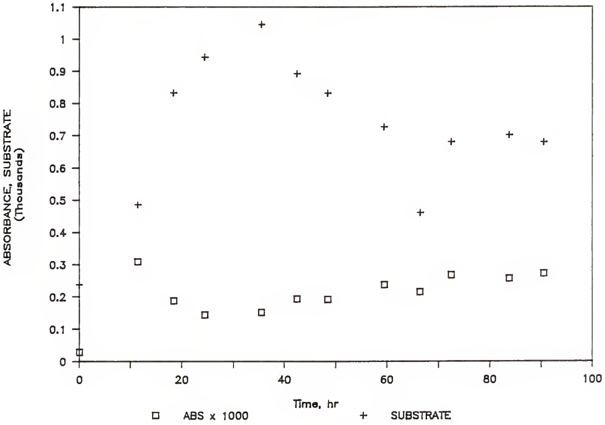


Figure 4-19. Absorbance and substrate concentrations for batch 13, continuous operation. Dilution rate 0.28 hr^{-1}. Inlet substrate 2000 mg/l.

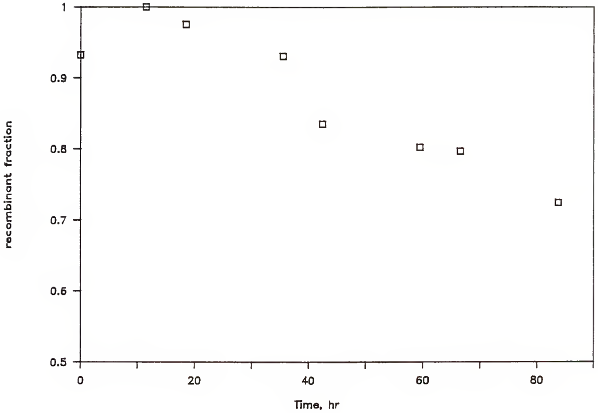


Figure 4-20. Change in recombinant fraction for continuous operation, batch 13. Dilution rate 0.28 hr^{-1}.

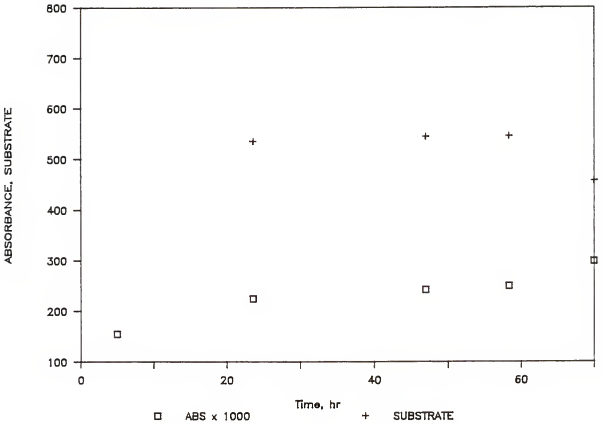


Figure 4-21. Absorbance and substrate concentrations for batch 14, continuous operation. Dilution rate 0.2 hr^{-1}. Inlet substrate 2000 mg/l.

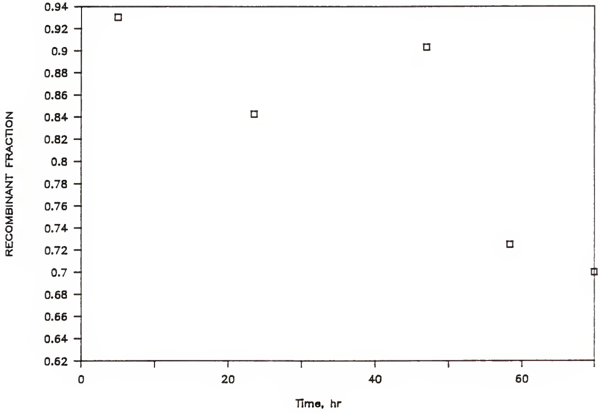


Figure 4-22. Change in recombinant fraction for continuous operation, batch 14. Dilution rate 0.2 hr^{-1}.

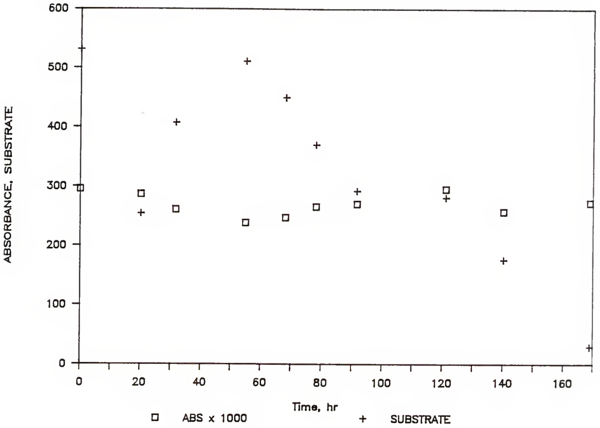


Figure 4-23. Absorbance and substrate concentrations for batch 15, continuous operation. Dilution rate 0.2 hr^{-1}. Inlet substrate 2000 mg/l.

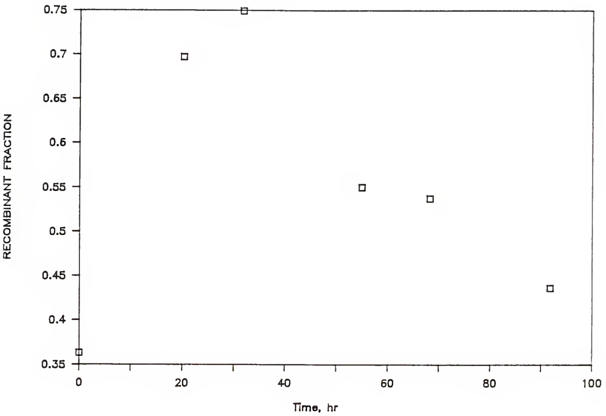


Figure 4-24. Change in recombinant fraction for continuous operation, batch 15. Dilution rate 0.2 hr^{-1}.

observation. The recombinant fraction shows a nice downward trend.

One of the most unexpected features of continuous operation of this culture is that the size of the cells must be very different under continuous and batch operation. The absorbance values for both types of experiments are comparable, but the numbers of cells show large differences. For example in batch 6, seen in Figure 4-10, the maximum absorbance values are between 0.200 and 0.250. The corresponding population values are in the 200 to 400 million cells/ml range. Compare this to batch 10, Figure 4-14. Again the absorbance is in the 0.200 to 0.250 range, but the corresponding numbers are less than 20 million cells/ml. These large differences cast doubt on whether results of batch experiments can be extended to continuous work, especially for the early batches where only cell numbers were available.

Batches 12 through 15 all demonstrate the typical behavior of a mixed recombinant continuous culture operating to steady-state. The fraction of recombinant bacteria in the population does decrease with time as expected. This result leads to the question of whether this behavior can be reversed through appropriate periodic operation as the theoretical work of previous chapters suggests.

The results of much of the work carried out in batch suggested that the reversion phenomenon required a significant time span to express itself with differences in the observed growth rate and the fraction of recombinant cells in the population. These continuous runs demonstrate that the reversion does indeed occur, with the most likely primary effect being the growth rate difference that further changes the population with time. Once a significant fraction of the cells are wild type, then the reversion phenomenon is overshadowed by growth rate differences and fails to make much more of a significant contribution to the increase in wild type. Thus the problem basically becomes the contamination problem discussed in chapter 2 with the difference that no extinction of wild type is possible. While a rapid reversion phenomenon would have been more interesting to study, this slowly reverting system is a good choice for demonstrating the effect of periodic operation on genetically engineered strains and for the general mixed population problem at the same time. Having one of the strains contain a simple antibiotic marker on the plasmid allows easy differentiation between two competing organisms, much easier than differentiation based on differences such as cell size.

4.6 Periodic Operation

The final fate of a culture of recombinant bacteria under continuous operation is the replacement of the original recombinant strain for its more efficiently growing plasmid-free counterpart. The objective here is to see if this trend can be reversed by implementing periodic variation of the environmental conditions in the chemostat.

The actual shift in a population from dominance by one strain to another was indeed illustrated in the previous section. In response to this replacement of one type of strain by another less desired organism, a method was investigated in Chapters 2 and 3. This method was to involve the periodic switching from one level of inlet substrate concentration to another level and back again in a square wave fashion. It was found that this method could reverse the trend of reversion from the desired to the undesired strain for certain operating conditions. It was also found that the method was not specifically limited to a particular model form or a particular periodic input. It was found that the desired response could be obtained not only by inlet substrate concentration switching, but also through implementing a square wave in the system dilution rate in simulations with the Monod model. The latter method was chosen for reasons of equipment limitations and simplicity of operation.

The periodic experiments carried out were intended for a single purpose, to determine if the final fate of a recombinant strain, extinction, could be avoided. As mentioned earlier the particular type of periodic operation carried out involved periodic switching between two levels of dilution rate. The lower dilution rate value was always set to zero. This was easily done by simply having a computer interfaced controller turn the feed pump off at the appropriate times. The load cell was used to maintain the system's constant volume (by switching the harvest pump off). At some later time, the computer switched the feed pump back on and the harvest pump turned on shortly thereafter.

The periodic experiments were carried out so that the total amount of medium fed to the system would be equal to the amount used for the continuous base case which was run at a dilution rate of 0.2 hr^{-1}. The inlet substrate concentration, s^0, for all periodic runs was set at 2000 mg/l. The desired upper value of the dilution rate was ideally set to 0.4 hr^{-1}. There was some difficulty in setting actual flow rates through the system because of the way the computer controlled the flow rate and the safety features of the load cell which would prevent the reactor from running dry.

In Figure 4-25 the effect of periodic operation is indeed seen to reverse the trend of reversion to wild type. This run was carried out by cycling in dilution rate between zero and 0.4 hr^{-1} at a cycling period of 2 hours. The system was started at a small initial value of recombinants in the population so that any positive effect would quickly

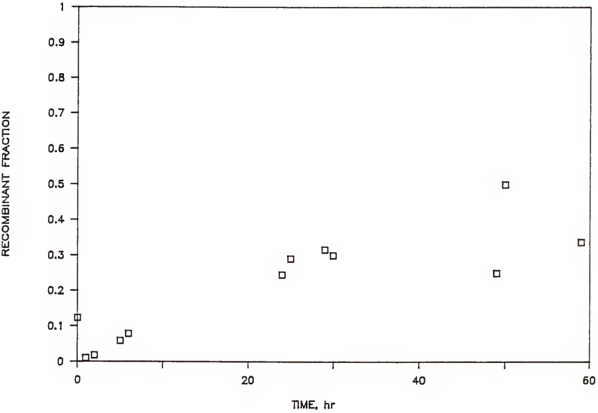


Figure 4-25. Change in recombinant fraction for periodic operation, batch 16. Cycling in dilution rate between 0.0 and 0.4 hr^{-1}. Cycling period is 2 hours. Inlet substrate 2000 mg/l.

manifest itself. There may be some questions about the actual recombinant fraction, but the values of the total population clearly drop while those of recombinant numbers definitely go up (see Figures 4-26 and 4-27). The numbers of cells of each type were always determined at integer multiples of the half period rather than during a period.

A second periodic run, see Figure 4-28, illustrates the same reversion in the trend of plasmid loss for a period of 4 hours. The dilution rate for this experiment was varied between zero and 0.45 hr^{-1}. These periodic runs prove that it is possible to reverse the effect of plasmid segregational instabilities in recombinant populations by a simple environmental manipulation.

4.7 Conclusions

The primary conclusion of the preceding experimental sections is that it is indeed possible to use simple periodic operation to prevent the expected extinction of a plasmid containing microorganism in continuous culture. The particular system that was studied, a mixed population of Escherichia coli containing a plasmid conferring antibiotic resistance and the resulting wild type counterpart, exhibited the desired properties. The batch experiments demonstrated that the recombinant strain did indeed grow more slowly than the wild type. It was also found that the

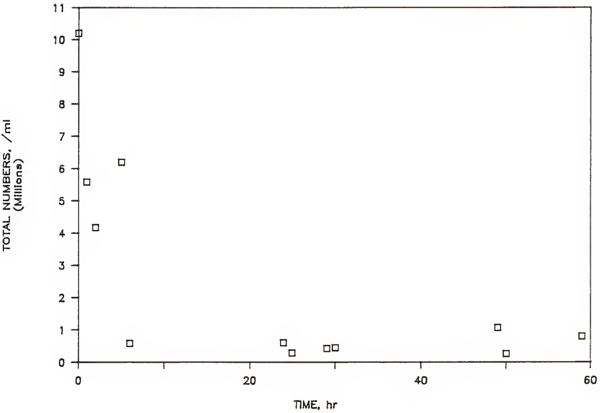


Figure 4-26. Change in total population for periodic operation, batch 16.

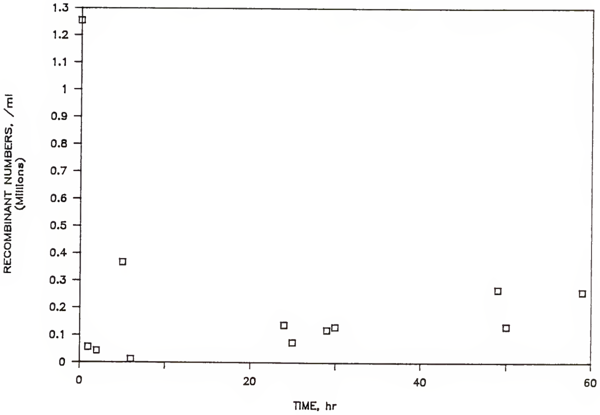


Figure 4-27. Change in recombinant population for periodic operation, batch 16.

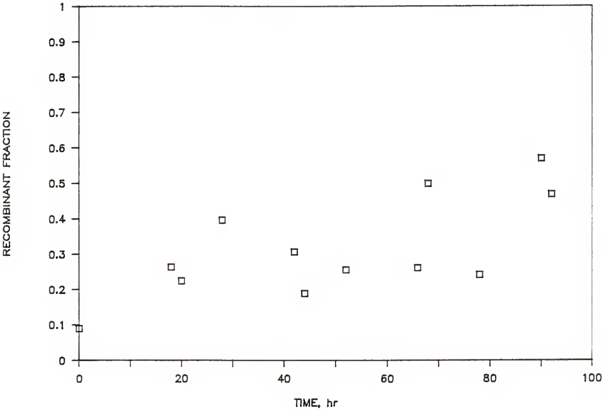


Figure 4-28. Change in recombinant fraction for periodic operation, batch 17. Cycling in dilution rate between 0.0 and 0.45 hr^{-1}. Cycling period is 4 hours. Inlet substrate 2000 mg/l.

plasmid-bearing strain is less responsive to sudden environmental changes.

The batch experiments also demonstrated that the fraction of recombinants in the population decreases with time and that the overall specific growth rate of the culture increases with time. It was discovered that the primary effect in the decrease of plasmid with time was the growth rate difference. The reversion phenomenon works on a much longer time scale than was observed in batch experiments.

The results of the parallel batch and fed-batch experiments demonstrated that no difference in plasmid retention in a population can be made by fed-batch operation within the range of conditions studied.

The continuous experiments demonstrated that the plasmid-bearing fraction of the culture did decrease with time as was expected. The periodic experiments demonstrated that the manipulation of a simple system operating parameter such as dilution rate can indeed produce effective growth rate differences between the two competing species and prevent the extinction of the more slowly growing one.

CHAPTER 5
DETERMINATION OF MEMORY FUNCTION FORM FROM TRANSIENT RESPONSES

In the previous theoretical development of the effects of periodic operation on a biological system where competition occurs between two organisms, Chapters 2 and 3, it was established that for a simple unstructured model with the addition of delay, the usual pattern of survival and extinction could be overturned. The effect of periodic variation of inputs to the system was examined as well as how the delay could be incorporated in a mathematical description of the system. The particular form of the delay selected was that first used by MacDonald (33). The effect that various other forms of delay have on system behavior has been examined by O'Neil (55), but a trial and error type procedure was used to determine which type of delay formulation would best describe the experimental data. This work will present a method to determine which type of delay could best be used to predict the behavior of a system under consideration. It will use transient response behavior to step changes and other dynamic variations in process operating variables. The first section will consider the theoretical development of the method. The next section will demonstrate the method by considering several example cases.

5.1 Theoretical Development

If the behavior of a biological system under steady-state conditions has been characterized by some simple unstructured model, then the specific growth rate has been expressed as a function of biomass and substrate concentration, $\mu(s,x)$. It is known that such unstructured models do not predict correctly transient responses. It is assumed that these non-steady-state responses have a specific growth rate of $\hat{\mu}(t)$ which can be expressed as a weighted average of the steady-state specific growth rates that would prevail at the previous concentrations of biomass and substrate, i.e.

$$\hat{\mu}(t) = \int_{-\infty}^{t} \mu[s(\tau),x(\tau)]\ F(t - \tau)\ d\tau \qquad (5\text{-}1)$$

where F(...) is a memory function which is appropriately normalized:

$$\int_{0}^{\infty} F(\tau)\ d\tau = 1. \qquad (5\text{-}2)$$

To derive the discrete form of this equation, let $t_i = ih$ and $t = Nh$. This leads to

$$\hat{\mu}_N = \hat{\mu}(t) = \sum_{i=-\infty}^{N-1} \int_{t_i}^{t_{i+1}} \mu[\tau]\, F(Nh - \tau)\, d\tau \qquad (5\text{-}3)$$

$$\approx \sum_{i=-\infty}^{N-1} \frac{h}{2} [\, \mu_i F_{N-i} + \mu_{i+1} F_{N-i-1} \,]. \qquad (5\text{-}4)$$

Setting h to 1.0 (amounting to the appropriate normalization of time) leads to the equivalent weighted substrate value at the Nth sampling instant

$$\hat{\mu}_N = \sum_{i=-\infty}^{N-1} \mu_i F_{N-i} + \frac{1}{2} \mu_N F_0 \qquad (5\text{-}5)$$

where μ_i is the value of $\mu(s,x)$ evaluated at the substrate and biomass concentrations of the system at time t_i. The discrete equation can be manipulated as follows. Let $j = N - i$. The summation then becomes

$$\hat{\mu}_N = \sum_{j=1}^{\infty} \mu_{N-j} F_j + \frac{1}{2} \mu_N F_0. \qquad (5\text{-}6)$$

The memory function weighs the previous values of substrate and biomass concentrations in determining the current value of the growth rate. It is evident that environmental conditions before some finite point in time do not make a contribution to the total. This leads to $F_j = 0$ for all $j > m \geq 0$, where m is the point in time before which the state of the culture (values of substrate and biomass concentrations) have no effect on the current value of the growth rate. This leads to an expression for the current growth rate of the form

$$\hat{\mu}_N = \sum_{j=1}^{m} \mu_{N-j} F_j + \frac{1}{2} \mu_N F_0 . \tag{5-7}$$

The problem that is then posed is to determine the necessary memory vector $\underline{F} = [F_0, F_1, \ldots, F_m]$ using measurements of the specific growth rate $\hat{\mu}_0, \hat{\mu}_1, \ldots, \hat{\mu}_N$ and the substrate and biomass measurements at times $1 - m, \ldots, N$. Letting M represent the N by (m + 1) matrix of steady-state growth rates that influence the current growth rate,

$$M = \begin{bmatrix} \mu_1 & \mu_0 & \cdots & \mu_{1-m} \\ \mu_2 & \mu_1 & \cdots & \mu_{2-m} \\ \vdots & \vdots & & \vdots \\ \mu_{m+1} & \mu_m & \cdots & \mu_1 \\ \mu_{m+2} & \mu_{m+1} & \cdots & \mu_2 \\ \vdots & \vdots & & \vdots \\ \mu_N & \mu_{N-1} & \cdots & \mu_{N-m} \end{bmatrix} \tag{5-8}$$

and $\underline{\hat{\mu}}$ the vector of measured specific growth rates

$$\underline{\hat{\mu}} = [\hat{\mu}_1, \hat{\mu}_2, \ldots, \hat{\mu}_N]. \tag{5-9}$$

The linear system to be solved for finding $\underline{F}$ can be represented as

$$M \, \underline{F} = \underline{\hat{\mu}}, \tag{5-10}$$

assuming that $N \geq m + 1$. The system above has the following least squares solution for the memory function

$$\underline{F} = (M^T \, M)^{-1} \, M^T \; \underline{\hat{\mu}} \; . \tag{5-11}$$

5.2 Illustration of the Method for Memory Function Determination

The very simple system that will be considered first in this section will be a system where the steady-state behavior of the specific growth rate has been found to be proportional to residual substrate concentration (30), $\mu = \delta s$. If the system's transient behavior can be predicted by the delay attributed to MacDonald, a memory function of the form $F = \alpha \exp(-\alpha \, \tau)$ as discussed in Chapters 2 and 3, then a system of the following form results:

$$d\mu/dt = \alpha\ (\delta s - \mu) \tag{5-12}$$

or letting $\mu = \delta z$ one gets

$$dz/dt = \alpha\ (s - z) \tag{5-13}$$

where δ is the proportionality constant between concentration and growth rate. For this case the memory function is

$$F(\tau) = \alpha\ \exp(-\alpha\ \tau), \tag{5-14}$$

an exponentially decaying function.

The simplest problem that can be considered involves a single step change in s from steady-state conditions. The F_i values are weighting factors that will sum to 1.0 (discrete equivalent of eqn. (5-2)) for sufficiently large values of N. The general form of this problem requires a least squares solution since the amount of data typically available overspecifies the system. It was found that with a single step change in s, the addition of data to matrix M in excess of the number of F_i's being solved for causes the quality of the estimated F_i's to deteriorate. This results because all rows in the M matrix numbered greater then m+1 are identical yet have different corresponding $\hat{\mu}$ values.

The (N x N) problem would be set up as follows:

$$\begin{bmatrix} s_1 & s_0 & \cdots & s_0 \\ s_1 & s_1 & \cdots & s_0 \\ s_1 & s_1 & \cdots & s_0 \\ \vdots & \vdots & & \vdots \\ s_1 & s_1 & \cdots & s_1 \end{bmatrix} \begin{bmatrix} F_0/2 \\ F_1 \\ F_2 \\ \vdots \\ F_{N-1} \end{bmatrix} = \begin{bmatrix} z_1 \\ z_2 \\ z_3 \\ \vdots \\ z_N \end{bmatrix} . \qquad (5\text{-}15)$$

Solving for F_i yields

$$F_{N-1} = \frac{z_N - z_{N-1}}{s_1 - s_0} , \quad F_{N-2} = \frac{z_{N-1} - z_{N-2}}{s_1 - s_0} , \quad \ldots \qquad (5\text{-}16)$$

$$F_2 = \frac{z_3 - z_2}{s_1 - s_0} , \quad F_1 = \frac{z_2 - z_1}{s_1 - s_0} , \quad F_0 = 2\left(\frac{z_N}{s_1} - \frac{z_N - z_1}{s_1 - s_0} \right) .$$

The value of all F_i's other than F_0 is constant for any value of N.

From an initial steady state where z = s, a step change was made in s from 100 to 200. An α value of 16 was selected and the system measured values were calculated at a delta t of .01 time units. Figure 5-1 shows the F_i values that result from solving the (25 x 25) case. For the memory function used in this system, an F_0 value of 0.16 is expected. The generated value of 0.314 is higher than was expected. This is an artifact caused by the way that the integral in eq. (5-3) was approximated in order to generate the discrete form of the equation. The trapezoidal rule used to evaluate the integral produced the $\mu_N F_0/2$ term that

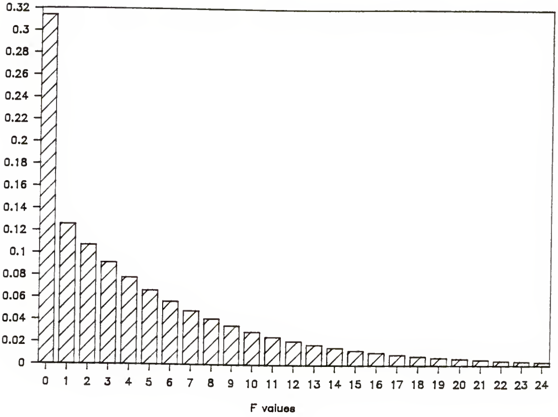


Figure 5-1. Memory function weighting factors for step change in s. System: dz/dt = α (s - z). Change in s from 100 to 200, α = 16.0, delta t = 0.1.

causes the discrepancy. If the division by 2 is taken into account when solving this problem, a value for F_0 of 0.157 results. The integral can be evaluated in ways other than with the trapezoidal rule. If the integral is approximated by multiplying the $\mu(t_{i+1})$ value (the value at the end of the interval rather than the mean value between ends) by the interval width, a slightly different discrete equation results. All F_i values are the same as before except for F_0 which now has the value of one-half its previous value, 0.157. For either case, in the limit as $N \rightarrow \infty$, $F_{N-1} \rightarrow 0$. Another source of this discrepancy involves the nature of the step change itself. A sudden drastic change occurs at time zero and this is reflected in the first point. In the cases which follow where s changes more slowly, this discrepancy does not occur.

Consider now the case where s varies sinusoidally i.e. $s = 50 + 25 \sin(4t)$. For $\alpha = 1.0$ and a delta t of 0.1, this system has the F_i values seen in Figure 5-2. If s varies as $s = 50 + 25 \sin(2t)$ and a delta $t = 0.2$ is used as the sampling interval, a different set of F_i values results (see Figure 5-3). Both sets will generate the same continuous memory function values if divided through by their respective delta t values.

For cases where a transient s value is being input to the system, all F_i values change as the number of F_i's increases. The effect on F_i with increases in N is

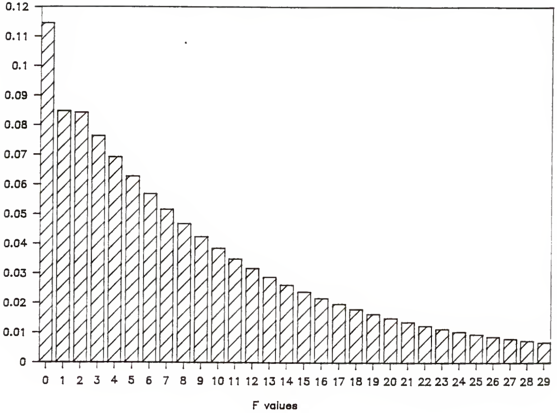


Figure 5-2. Memory function weighting factors for sinusoidal variation in s. s = 50 + 25 sin(4t), α = 1.0, delta t = 0.1.

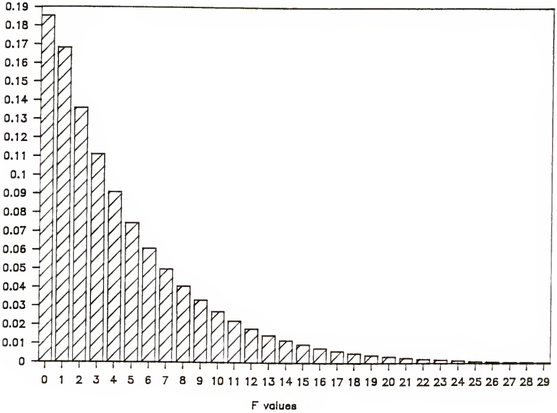


Figure 5-3. Memory function weighting factors for sinusoidal variation in s. s = 50 + 25 sin(2t), α = 1.0, delta t = 0.2.

demonstrated in Figure 5-4. As N increases the F_i values appear to converge. For Figure 5-4, the system is as as follows: s = 50 + 25 sin(2t), $\alpha = 1$, and delta t = 0.2. For small N the F_i have an occasional negative among them (indicating that insufficient quantities of data have been considered), but as N increases the individual values converge.

Other systems could have the delay incorporated in more complex ways and be manipulated indirectly. This would be a model of the type discussed in section 5.1 where the specific growth rate of the culture is a function of the substrate and biomass concentrations in the system, $\mu(s,x)$. One such system would be the Monod model with the addition of delay. Such a system of equations would be as follows:

$$\begin{aligned} \frac{dx}{dt} &= \frac{\mu_m z}{K + z} x - D x \\ \frac{ds}{dt} &= -\frac{\mu_m s}{Y (K + s)} x + D (s^0 - s) \qquad (5\text{-}17) \\ \frac{dz}{dt} &= \alpha (s - z). \end{aligned}$$

This is a set of model equations of the type discussed in Chapter 2, but for a pure rather than a mixed population of bacteria. Also in typical continuous operation in a chemostat the operating parameters are dilution rate and

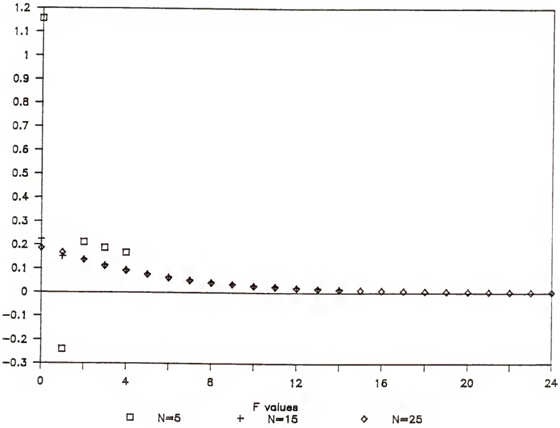


Figure 5-4. Effect on memory function weighting factors as N increases. s varies as s = 50 + 25sin(2t), α = 1.0, delta t = 0.1.

inlet substrate concentration. The actual bulk concentration of glucose in the system cannot be directly changed. The parameters for this system were $\mu_m = 0.5$, $K = 50$, $Y = 0.5$, $s^0 = 100$, $\alpha = 1.0$, and delta $t = 0.1$. The dilution rate in this system was changed from an initial value of 0.25 to 0.2. The F_i's are shown in Figure 5-5. It can be seen in Figure 5-6 that a slight difference in the memory function is determined for a step up in D (D = 0.3) rather than a step down. For refined enough sampling one would expect the two sets of F_i's to coincide. Also of interest is the effect on F_i of a step up in the s^0 value ($s^0 = 200$) compared to a step up in D, see Figure 5-7. Again there is a slight discrepancy that is expected to go away with better sampling.

In summary, given a system whose steady-state behavior can be predicted using an unstructured model, a method has been developed to determine the weighting factors relating current growth rates and previous environmental conditions. The values of these weighting factors indicate the form of delay memory function that best describes the system's responses under transient conditions.

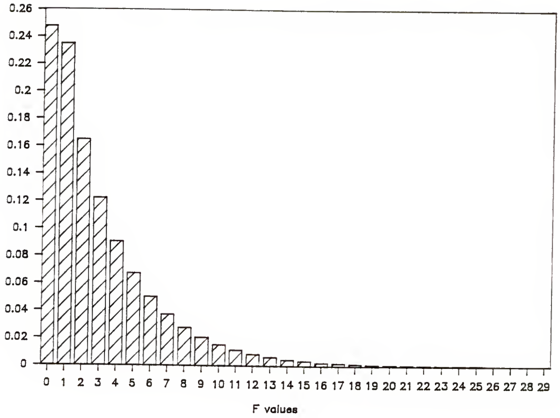


Figure 5-5. Memory function weighting factors for Monod model with delay for step down in dilution rate. Dilution rate changes from 0.25 to 0.2, α = 1.0, delta t = 0.1.

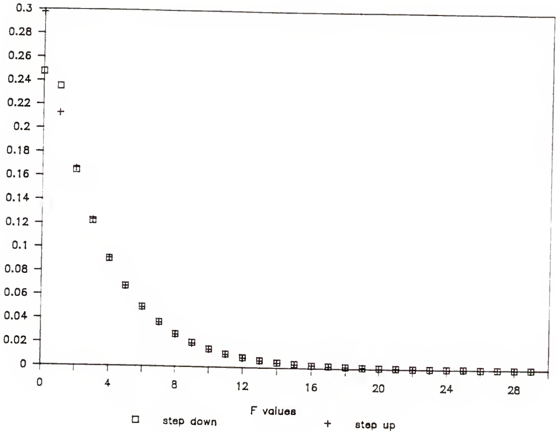


Figure 5-6. Memory function weighting factors for Monod model with delay for step down in dilution rate versus step up. Step down, D = 0.2. Step up, D = 0.3. α = 1.0, delta t = 0.1.

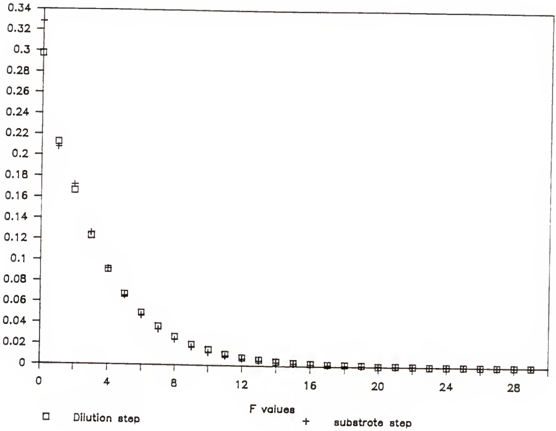


Figure 5-7. Memory function weighting factors for Monod model with delay for step up in dilution rate versus step up in inlet substrate concentration. Dilution rate from 0.25 to 0.3. Inlet substrate from 100 to 200.

CHAPTER 6
SUMMARY

It has been apparent since the very first chemical process was developed that there were many alternate ways to produce the compounds needed for an industrial society. Many processes that were originally batch have been displaced by more efficient continuous processes. For many continuous operations, periodic operation has proved still more beneficial in meeting particular performance objectives. As microorganisms are increasingly used to produce biochemicals, methods optimizing production objectives become more important. Just as certain continuously operating chemical systems have benefitted from periodic operation, it is expected that this will be true for certain classes of biochemical systems as well.

The problem of competing cultures under continuous operation has been studied by many workers. The general conclusion of their work is that a single organism, the most efficient one, will ultimately dominate a mixed competitive culture under steady-state conditions. The organism that dominates the steady-state culture is the one with the largest specific growth rate at the given operating conditions. In certain cases there are different ranges of operating conditions that favor each of the two competing organisms. It has been shown for these cases that alternating between two sets of conditions, e.g. pH or temperature, can favor first one organism and then another. The net result of this type of operation is the coexistence of both organisms.

The first problem that was considered in this work was a competitive mixed culture where the growth rate of the desired organism is always less than that of the undesired organism for any constant environmental conditions. It was shown that the final fate of such a competitive culture cannot be affected by periodic operation if simple steady-state based unstructured models accurately predict transient responses to environmental changes. For most systems the predictive powers of these simple models are poor. One way to correct this problem is the incorporation of a time delay in the growth expressions. For those cases where inclusion of time delay is necessary to predict transient behavior, it was shown that periodic operation can be used to prevent the extinction of the slower growing species provided the faster growing species also responds to environmental changes more rapidly than the more slowly growing species. This is accomplished by using cycling in reactor operating parameters to obtain differences in the effective growth rates of the two species that benefit the desired slower growing slower adapting species.

This work was extended to the specific case of recombinant organisms and the competitive situation that arises due to the segregational instability of the engineered strain. Over time, a fraction of the pure recombinant culture loses its plasmid and reverts to wild type. This wild strain is faster growing than its recombinant counterpart. The final result of this instability during continuous operation is the extinction of the recombinant strain. The objective of this portion of the study was to determine if periodic operation could reverse the trend of plasmid loss that occurs during continuous operation. It was again found that in the absence of time-delay in the response to transient conditions no benefit from periodic operation can be obtained. However if time-lag exists in the growth rate adaptation to environmental changes, then it is possible to prevent the plasmid-bearing strain from becoming extinct if the wild strain responds to environmental changes more rapidly than the recombinant strain.

The theoretical results of periodic operation of a system of recombinant bacteria were experimentally tested to see if the trend towards plasmid loss in continuous culture could indeed be reversed. A system of recombinant

Escherichia coli containing a plasmid conferring antibiotic resistance (pbr322) was selected to test the hypothesis.

Batch experiments demonstrated that the selected system exhibited the properties needed to expect possible plasmid retention under periodic operation. The two most important factors discovered were that the wild strain did grow more rapidly than the recombinant strain and that the wild type responds more rapidly to environmental changes. It was also found that this system experiences the expected decrease of recombinant fraction with time when the inoculum for a batch run contains a mixed population. Also observed was that the culture overall specific growth rate increases as the plasmid-bearing fraction decreases. In addition, it was seen that the primary effect in the decrease of plasmid-bearing fraction with time is the growth rate difference rather than the reversion phenomenon.

A series of parallel batch and fed-batch runs were carried out to determine if fed-batch operation could influence plasmid retention. It was found that no differences between the two types of operation could be detected with respect to loss of plasmid for the conditions used in this study.

Continuous runs were carried out to demonstrate the loss of plasmid with time and to act as a base case with which to compare the success or failure of periodic operation. The expected plasmid loss during continuous operation was demonstrated for several runs.

Periodic operation was carried out by cycling in system dilution rate and it was found that periodic operation can indeed reverse the trend of plasmid loss normally seen in steady-state continuous operation.

A method was also developed to determine the delay formulation that could best predict transient responses to environmental changes for a system whose steady-state behavior has been previously modeled.

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BIOGRAPHICAL SKETCH

Mark Lee Stephens was born in the stagnant backwater town of Anderson, South Carolina, on September 11, 1962. A forgettable childhood preceded his escape to Clemson University in August 1980 where he entered the chemical engineering program. Graduating summa cum laude with a Bachelor of Science in May 1984, Mark completed the next phase of his escape from South Carolina by journeying to the University of Florida. He entered the graduate program in chemical engineering in August, 1984, and received a Master of Science degree the following August. As a doctoral student at the University of Florida, the next several years saw him in servitude to a strain of recombinant bacteria who really did not care that he was working on his doctorate.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gerasimos Lyberatos, Chair
Associate Professor of
Chemical Engineering

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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